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**Ms Luisa Maria Rosaria Napolitano, master's degree (cum laude)
awarded in the March 29, 2007 at the University "Federico II" of
Naples, Italy.**

**The TRIM (TRipartite Motif) Family as a Novel Class of
Ubiquitin E3 Ligases**

**A thesis submitted to the Open University in fulfillment of the
requirements for the degree of Doctor of Philosophy in Life and
Biomolecular Sciences**

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ABSTRACT

Proteins that belong to TRIM family are characterized by the presence of the tripartite motif module, composed of a RING domain, one or two B-box domains and a Coiled-coil region. TRIM proteins are involved in several cellular processes such as apoptosis, cell cycle regulation and viral response. The aim of my project was to study the involvement of TRIM proteins in the ubiquitylation process, a versatile post-translational modification mechanism used by eukaryotic cells mainly to control proteins levels through proteasome-mediated proteolysis. In particular, the presence of the RING domain and experimental data suggested a possible TRIM role as Ubiquitin Ligases (E3), the component of the ubiquitylation cascade responsible for the transfer of Ubiquitin to the specific target.

A condition for being an E3 is the interaction with another component of the ubiquitylation cascade, the Ubiquitin Conjugating Enzymes (UBE2). Therefore, I tested the interaction between 26 UBE2 enzymes and 42 TRIM proteins. I observed that the majority of the TRIM proteins tested interact with UBE2 enzymes and I also found a general preference of the TRIM proteins for the D and E classes. Two important exceptions were observed: TRIM9-UBE2G2 and TRIM32-UBE2V1/2 specific interactions. Furthermore, representative interactions were confirmed and I also demonstrated that the TRIM E3 activity is only manifest with the UBE2 they interact with. For most specific interactions I could also observe subcellular co-localisation of the TRIM involved and its cognate UBE2 enzyme suggesting that the specific selection of TRIM-UBE2 pairs has physiological relevance.

In addition I found that almost all TRIM proteins tested interacted with UBE2I that is the specific E2 enzyme involved in modification with SUMO, a Ubiquitin-like peptide. Consistently, representative interactions were confirmed and for a subset of TRIM proteins I demonstrated the

involvement in the SUMOylation pathway suggesting a possible cross-talk between ubiquitylation and SUMOylation.

TABLE OF CONTENTS

ABSTRACT	1
TABLE OF CONTENTS	3
TABLE OF FIGURES	6
CHAPTER 1: INTRODUCTION	8
1.1 UBIQUITYLATION	8
1.2 UBIQUITYLATION MACHINERY: E1, E2 AND E3	11
1.2.1 Ubiquitin-Activating Enzyme (E1).....	11
1.2.2 Ubiquitin Conjugating Enzyme (UBE2)	12
1.2.3 Ubiquitin Ligase (E3)	14
1.3 ROLE OF UBIQUITYLATION	22
1.3.1 The Ubiquitin-proteasome system.	25
1.3.2 Monoubiquitylation	31
1.4 UBIQUITIN-LIKE MODIFICATION	34
1.5 THE TRIM PROTEIN FAMILY	40
1.5.1 TRIM domain structure	43
1.5.2 TRIM proteins as E3 Ubiquitin Ligases	48
CHAPTER 2: AIM OF THE PROJECT	52
CHAPTER 3: MATERIALS AND METHODS	54
3.1 CLONING STRATEGY	54
3.1.1 RNA extraction	54
3.1.2 Production of insert DNA fragments	55
3.1.3 DNA restriction.....	56
3.1.4 DNA ligation	57
3.1.5 DNA transformation	57
3.1.6 Purification of plasmid DNA	58

3.2 INTERACTION MATING	59
3.2.1 Cloning the Ubiquitin Conjugating Enzyme cDNAs into vectors for expression in yeast	61
3.2.2 DNA transformation in yeast	66
3.2.3 Yeast Cell lysates	67
3.2.4 Interaction mating assay	67
3.3 CELL CULTURE AND IMMUNOBLOT	70
3.3.1 Cloning the UBE2 cDNAs in vectors suitable for transfection in HEK293 and HeLa cells.	70
3.3.2 Cell Culture and transient transfection	72
3.3.3 Immunoblot analysis	72
3.4 IMMUNOFLUORESCENCE	73
3.5 MBP-TRIM PROTEIN EXPRESSION AND PURIFICATION	74
3.5.1 Cloning the TRIM cDNAs in vectors suitable for expression in E.coli	74
3.5.2 MBP-TRIM protein expression and purification	76
3.6 MBP PULL-DOWN ASSAY	77
3.7 IN VITRO UBIQUITYLATION ASSAYS	77
3.8 IN VIVO UBIQUITYLATION ASSAYS	78
3.9 IN VITRO SUMOYLATION ASSAYS	78
CHAPTER 4: RESULTS	79
4.1 PRELIMINARY SCREENING OF TRIM PROTEINS AND UBIQUITIN CONJUGATING ENZYMES INTERACTIONS PERFORMED THROUGH INTERACTION MATING.	79
4.1.1 Interaction Mating assay	79
4.1.2 Western Blot analysis of both TRIM and UBE2 protein expression in yeast cell	83
4.1.3 The Interaction Mating assay between UBE2 enzymes and TRIM proteins	85
4.1.4 TRIM proteins interact with UBE2 enzymes through their RING domain	89
4.2 VALIDATION OF THE INTERACTION BETWEEN TRIM AND UBE2 PROTEINS THROUGH MBP PULL-DOWN ASSAYS.	91
4.2.1 MBP pull-down assay	91

4.2.2 <i>Expression and purification of MBP-TRIM proteins</i>	92
4.2.3 <i>MBP pull-down experiments</i>	95
4.3 <i>IN VITRO</i> UBIQUITYLATION ASSAY	97
4.4 <i>IN VIVO</i> UBIQUITYLATION ASSAY	103
4.5 SPECIFIC TRIM-UBE2 BINDING OCCURS IN HeLA CELLS	106
4.6 TRIM PROTEINS INVOLVEMENT IN SUMOYLATION PATHWAY	112
4.6.1 <i>TRIM proteins interaction with UBE2I</i>	112
4.6.2 <i>In vitro SUMOylation assays</i>	118
CHAPTER 5: DISCUSSION	121
BIBLIOGRAPHY	135

TABLE OF FIGURES

FIGURE 1 MODEL OF E3 PROTEIN-MEDIATED UBIQUITIN OR UBIQUITIN LIKE MODIFICATION.....	10
FIGURE 2 A COMPARISON OF UBIQUITIN LIGASES.....	21
FIGURE 3 EXAMPLES OF DIFFERENT UBIQUITIN CHAINS.	24
FIGURE 4 FORMATION OF THE 26S PROTEASOME	28
FIGURE 5 THE SUMOYLATION PATHWAY	39
FIGURE 6 TRIM PROTEINS HOMOMULTIMERIZE AND ASSOCIATE WITH SPECIFIC SUBCELLULAR STRUCTURES.....	42
FIGURE 7 RING, B-BOX1 AND B-BOX2 CONSENSUS SEQUENCES	46
Figure 8 A schematic representation of the TRIM proteins identified.....	47
FIGURE 9 A SCHEMATIC REPRESENTATION OF PSH18-34	60
FIGURE 10 THE PEG202 VECTOR.....	64
FIGURE 11 THE PJG4-5 VECTOR	65
FIGURE 12 THE INTERACTION MATING TECHNIQUE	69
FIGURE 13 THE pCDNA3 VECTOR.....	71
FIGURE 14 THE PMAL-C2X VECTOR	75
FIGURE 15 SCHEMATIC REPRESENTATION OF THE INTERACTION MATING	82
FIGURE 16 WESTERN BLOT ANALYSIS OF TRIM AND UBE2 PROTEINS EXPRESSED IN YEAST CELLS.....	84
FIGURE 17 EXAMPLES OF INTERACTION MATING	87
FIGURE 18 TWO-INTERACTION MATING PANELS SHOWING EITHER TRIM18 OR TRIM32 DOMAIN DELETED MUTANTS	90
FIGURE 19 COOMASSIE GEL STAINING OF HIGH-LEVEL EXPRESSION AND PURIFICATION OF TRIM PROTEINS FUSED TO MBP	93

FIGURE 20 MBP PULL-DOWN ASSAY CONFIRMS UBE2 AND TRIM PROTEINS SPECIFIC INTERACTIONS	96
FIGURE 21 CONTROL OF THE <i>IN VITRO</i> UBIQUITYLATION ASSAY	100
FIGURE 22 TRIM PROTEINS ACT AS UBIQUITIN E3 LIGASES <i>IN VITRO</i> USING THE INTERACTING UBE2 ENZYMES	101
FIGURE 23 IMMUNOBLOTS USING ANTI-MBP ANTIBODY	102
FIGURE 24 E3 LIGASE ACTIVITY OF TRIM PROTEINS IN HEK293 CELLS	105
Figure 25 TRIM9 co-localizes with UBE2G2 in cytoplasmic speckles.....	108
FIGURE 26 TRIM32 CO-LOCALIZE WITH UBE2N, V1, V2 PROTEINS	110
FIGURE 27 TWO INTERACTION MATING PANELS SHOWING EITHER RING-LESS TRIM20 OR TRIM18 DOMAIN DELETED MUTANTS	116
FIGURE 28 MBP-PULL DOWN ASSAYS CONFIRM THE INTERACTIONS OBSERVED BETWEEN TRIM PROTEINS AND UBE2I ENZYME.....	117
FIGURE 29 <i>IN VITRO</i> SUMOYLATION ASSAYS	119
FIGURE 30 A PHYLOGENETIC TREE PRODUCED UPON THE ALIGNMENT OF THE 20 UBE2 PROTEINS TESTED	128
FIGURE 31 ALIGNMENT OF SOME TRIM PROTEINS RING MOTIF	129

CHAPTER 1: INTRODUCTION

1.1 Ubiquitylation

Ubiquitylation is a form of post-translational modification of proteins in eukaryotes in which ubiquitin, a highly evolutionary conserved 76-residue polypeptide, is linked to target proteins (Hershko, Heller et al. 1983). Ubiquitylation of proteins is achieved through an enzymatic cascade involving Ubiquitin Activating Enzyme (E1), Ubiquitin Conjugating Enzyme (E2) and Ubiquitin Ligase (E3). An additional Ubiquitylation factor, whose name is E4, can sometimes induce the synthesis of long multiubiquitin chains (Koegl, Hoppe et al. 1999). The fate of the ubiquitylated proteins depends on the type of modification received. During ubiquitylation, ubiquitin chains or single ubiquitin molecules are linked to target proteins, giving rise to poly- or monoubiquitylation respectively. Polyubiquitylation generally targets proteins for degradation by the proteasome; instead, monoubiquitylation is implicated in endocytosis, endosomal sorting and histone regulation (Sun and Chen 2004). However, in some cases (i.e. K63 polyubiquitin chain) polyubiquitylation also has non-proteasomal regulatory functions like targeting proteins to nucleus, cytoskeleton and endocytic machinery, or modulating enzymatic activity and protein-protein interactions (see paragraph 1.3).

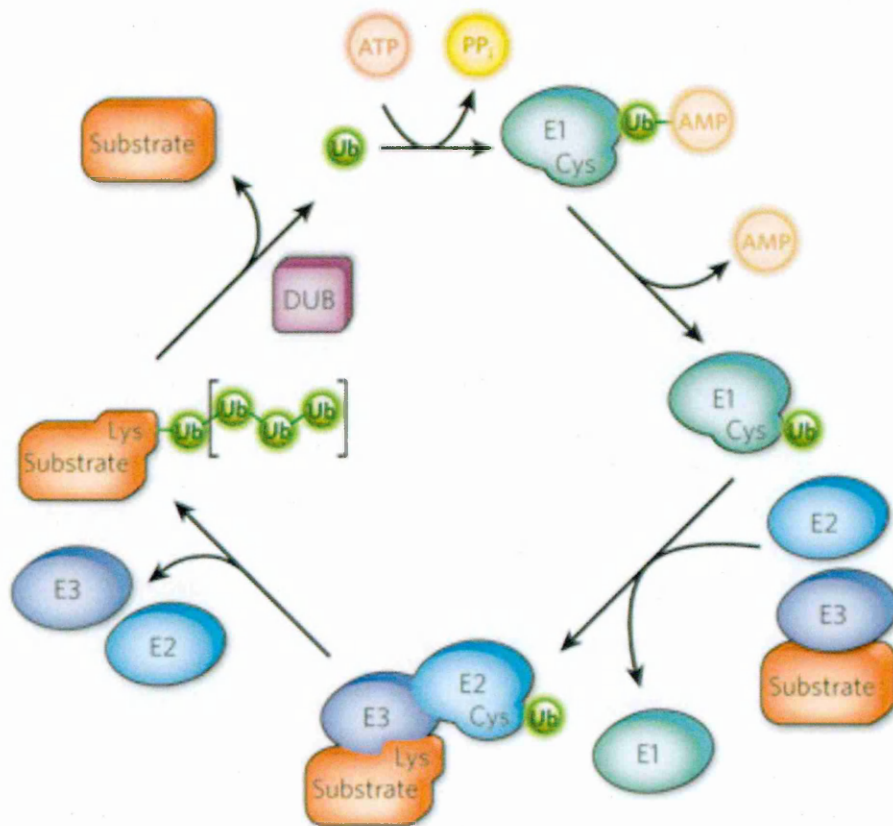
The ubiquitylation process starts with the synthesis of ubiquitin as a larger peptide that must be processed to reveal the C-terminal Glycine (Gly) residue, which will be the site of attachment of target molecules. This processing is carried out by Deubiquitylation enzymes (DUB) that also remove ubiquitin from modified substrate (Wilkinson 1997). The E1 enzyme initiates the cascade by catalyzing adenylation of the ubiquitin, by forming an acyl-phosphate linkage with AMP. The catalytic cysteine (Cys) of E1 attacks Ubiquitin~AMP complex, releasing AMP and forming a thioester linkage between the E1 catalytic Cys and the ubiquitin C-terminus (**Figure 1**).

Ubiquitin is then transferred to an E2, again through a thiol-ester linkage. E3s, which are primarily responsible for assuring specificity to ubiquitin conjugation, interact with the ubiquitin-charged E2 and the substrate, facilitating the formation of isopeptide bonds between the C-terminus of ubiquitin and lysines (Lys) on the target protein (**Figure 1**).

To date, eukaryotic genomes have been found to encode two or at most a few E1s; a great number of E2s exists, at least 11 in yeast and over 35 in human (van Wijk and Timmers 2010). Instead, the diversity and number of proteins that are regulated by ubiquitylation predict the existence of 600-1,000 E3s (Ardley and Robinson 2005). This allows modification of many proteins in a highly specific manner, and such modifications are often under temporal and spatial control.

The ubiquitylation process creates specific and reversible switches between different functional states of a substrate protein, allowing the fine control of numerous cellular pathways.

Figure 1



(Hochstrasser 2009)

Model of E3 protein-mediated ubiquitin or ubiquitin like modification. The E3-E2 substrate unit can assemble to form larger complexes that may contain several ubiquitylation pathway components. The ubiquitylation or ubiquitin like modification factors are distributed within the cells as discrete compartments that participate in different pathways.

1.2 Ubiquitylation machinery: E1, E2 and E3

1.2.1 Ubiquitin-Activating Enzyme (E1)

The main function of the Ubiquitin-Activating Enzyme (E1) is to catalyse the adenylation of ubiquitin at the expense of an ATP molecule. The resulting adenylated-ubiquitin is transferred to the active site cysteine (Cys) of E1 through the formation of a thioester bond; then, ubiquitin is transferred to the active site Cys of the Ubiquitin Conjugating enzyme (UBE2), the next enzyme in the ubiquitylation cascade.

The E1 enzyme is highly conserved in plants (Hatfield, Callis et al. 1990), in humans (Handley, Mueckler et al. 1991) and in yeast where it plays a critical role since the deletion of yeast E1, *UBA1*, is lethal (McGrath, Jentsch et al. 1991). In human, two E1 enzymes are known to initiate ubiquitin conjugation: *UBA1* and *UBA6*. Even if they are distantly related (about 40% identity), they share a common structure. They contain three domains: an adenylation domain composed of two ThiF –homology motifs (Lake, Wuebbens et al. 2001), which binds ATP and Ubiquitin; the catalytic Cys domain (CCD), which is the acyl carrier for ubiquitin; the C-terminal ubiquitin-fold domain which binds the UBE2 enzymes. Interestingly, Jin et al. (Jin, Li et al. 2007) demonstrated that *UBA1* and *UBA6* interact with the UBE2 enzymes in a specific manner. They examined E1 specificity in UBE2 charging in a panel of 29 UBE2s and they found that some UBE2 interacted with both *UBA1* and *UBA6*, but some others interacted with either *UBA1* or *UBA6*.

In addition to their chemical roles in initiating ubiquitin conjugation cascade, E1 enzymes also establish specificity by matching ubiquitin or Ubiquitin-like peptide (UBL, see paragraph 1.4) with specific E2s. For examples *UBA1* enzyme is able to recognize the C-terminal tail residue 72 in ubiquitin and to distinguish it from the same residue of the other Ubiquitin-

like peptides (Arg in ubiquitin and, for example, Ala in NEDD8). Rules for how this specificity is achieved are only beginning to emerge, but it is clear that specificity is achieved at multiple levels (Schulman and Harper 2009).

Moreover, *UBA1* enzyme is regulated by phosphorylation and proposed roles of *UBA1* phosphorylation include increasing nuclear import and/or retention, and modulation of nucleotide excision repair during macrophage differentiation (Stephen, Trausch-Azar et al. 1996; Nospikel and Hanawalt 2006). Furthermore, distinct isoforms of *UBA1* display different subcellular localizations (Grenfell, Trausch-Azar et al. 1994) although the functions of different *UBA1* modifications and isoforms remain poorly understood.

1.2.2 Ubiquitin Conjugating Enzyme (UBE2)

The ubiquitin peptide, after being activated by E1, is transferred to a Ubiquitin Conjugating Enzyme (UBE2) cysteine (Cys) residue as a thioester and from there, in an E3-dependent manner, to the substrate (Hershko, Heller et al. 1983). Eleven UBE2 enzymes have been identified in the yeast genome (Ubc1-8, 10, 11, 13). Two additional enzymes, Ubc9 and Ubc12, are specific for the ubiquitin-like proteins Smt3 and Rub1, respectively (Glickman and Ciechanover 2002). Many more E2s have been described in higher organisms. Typically, in humans 38 different UBE2 proteins were found that are broadly grouped into four classes, all of which are distinguished by the presence of a Cys-catalytic core domain (UBC): class I enzymes consist of just the UBC domain; class II possess a UBC and a C-terminal extension; class III possess a UBC and an N-terminal extension; and class IV possess a UBC and both N- and C-terminal extensions. These extensions appear to be important for some subfamily function, including E2 localization and protein-protein interactions. Ubiquitin E2 variant (UEV) proteins also have a UBC domain but lack the active-site Cys residue (Sancho, Vila et al. 1998).

The first important task of an UBE2 protein is to ensure that it receives ubiquitin, but not related Ubiquitin-Like peptides (UBLs) on its active site. The only exception to this role is UbcH8/UBE2L6 since it can conjugate both ubiquitin and ISG15, an ubiquitin-like peptide (Durfee, Kelley et al. 2008). Although E1s and E2s for UBLs have a structure similar to those of the corresponding enzymes for ubiquitin, E2s for ubiquitin specifically interact with the two E1s of the ubiquitin pathway. Biochemical and structural analyses revealed that E2s bind E1s with high affinity only if the E1 is carrying their modifier. Moreover, the charging of an E1 with ubiquitin changes conformation of the E1 exposing cryptic E2 binding sites allowing the formation of the proper E1-E2 complex (Huang, Hunt et al. 2007)

After being charged with ubiquitin, E2s engage E3s to catalyse substrate ubiquitylation. E2 enzymes act via selective protein-protein interactions with the E1 and E3 enzymes and connect activation to covalent modification. By doing so, E2s differentiate effects on downstream substrates, either with a single Ub/UBL molecule or as a chain. Indeed, while E3s are involved in substrate selection, generally E2s are the main determinants for selection of the Lysine (Lys) to build different ubiquitin chains (Kim, Kim et al. 2007). It has been well established that for example the UBE2D family of E2s, UBE2G2, cdc34 (cell division cycle 34) and UBE2K mediate Lys48-linked polyubiquitin chain formation that mark protein for degradation via proteasome (Li, Tu et al. 2007) ; whereas, UBE2N and UBE2V1/V2 protein mediate the assembly of Lys63-linked poly-Ub chains that play a regulatory role in diverse signalling pathways in a non proteolytic fashion (McKenna, Spyrapopoulos et al. 2001)

Moreover, in addition to cycling between E1s and E3s, some E2s bind cofactors that influence their localization, activity or specificity. This is best understood for the yeast coupling of ubiquitin conjugation to ER degradation protein 1 (Cue1), a transmembrane protein of the ER that uses a C-terminal motif to recruit UBE2G2 (Chen, Mariano et al. 2006). The association with

Cue1 also increases the ubiquitylation activity of UBE2G2 and protects it from autoubiquitylation and proteasomal degradation. These properties allow Cue1 to focus UBE2G2 activity on substrates at the ER membrane and, moreover, Cue1 is required for UBE2G2-dependent ER-associated degradation (ERAD).

The nomenclature of the different E2 enzymes is confusing, and similar terms given to yeast and mammalian enzymes do not reflect functional or structural homology. When the first E2 genes were cloned researchers mostly used the form E2-nK (where n denotes the molecular weight of the E2) and UBCn in yeast or UBCHn in humans (where n corresponds to the order of discovery). Other E2s were labelled following their discovery in genetic or proteomic screens, without a reference to their E2 function, for example Huntingtin-interacting protein 2 (HIP2; also known as E2-25K, UBCH1 and UBE2K). As a result, E2s from different organisms bearing the same number are often not functionally related, and most E2s have multiple names. Thus human UBCH1 (Kaiser, Mansour et al. 1994) is not the human homolog of yeast Ubc1, but rather the homolog of yeast Ubc2/Rad6. Yeast ER Ubc6 and Ubc7 are not the homologs of human UbCH6 and UbCH7 that are soluble enzymes involved in targeting of soluble proteins in the cytosol (Nuber, Schwarz et al. 1996).

1.2.3 Ubiquitin Ligase (E3)

The Ubiquitin Ligases (E3) are responsible for bringing the ubiquitin-charged E2 enzymes, through binding, in close proximity to the specific substrate allowing the correct transfer of the ubiquitin moiety. The E3s act as single proteins or protein complexes that bind both the Ubiquitin-charged E2 and the substrate. In most cases (i.e., RING domain E3s, see below), the E3s serve as scaffold that bring both the E2s and the substrate. In other cases (i.e., HECT domain E3, see below), the activated ubiquitin is transferred from E2

to an internal cysteine (Cys) residue on E3 before conjugation of ubiquitin to an ϵ -NH₂ group of an internal lysine (Lys) residue in the target. Less commonly, ubiquitin is conjugated to the terminal amino group of the substrate (Ciechanover and Ben-Saadon 2004) or even to Cys side chains via a thioester bond (Cadwell and Coscoy 2005). For its activity, the E3 ubiquitin ligase uses protein-protein interaction domains outside the catalytic domain to bind the substrate.

Because of lack of significant homology among the ligases initially identified, it was thought that they belong to a large number of protein families. Recently, it has become clear that even though E3s are heterogeneous, they can be classified into two major groups, in relation to their architecture: HECT domain E3 enzymes and four classes of RING finger E3 ubiquitin ligases: the SCF, VBC and anaphase-promoting (APC) complexes, and single-polypeptide RING-finger E3 enzymes (**Figure 2**).

1. HECT domain E3

The HECT domain protein family was originally identified by sequence similarity to the C-terminal region of E6-AP, an E3 ubiquitin ligase required for ubiquitylation of the tumour suppressor protein p53 induced by the E6 oncoprotein of HPV virus (Huibregtse, Scheffner et al. 1995). In contrast to the RING domain E3s, HECT domain E3s directly catalyze protein ubiquitylation. Ubiquitin-charged E2 enzymes directly transfer ubiquitin to the active-site cysteine (Cys) within the HECT domain in a transthioylation reaction, preserving the high-energy ubiquitin thioester bond. Substrate ubiquitylation occurs by nucleophilic attack of the E3-ubiquitin thioester bond by a lysine side chain of the target protein, although the mechanism of polyubiquitylation is still unclear (Huibregtse, Scheffner et al. 1995).

Structural information is available for the HECT domains of human E6AP/Ube3A (Huang, Kinnucan et al. 1999), WWP1 (Verdecia, Joazeiro et al. 2003) and Smurf2 (Ogunjimi, Briant et al. 2005). All HECT E3s have an N-terminal domain, which varies among the different HECT domain proteins and is involved in specific substrate recognition (Schwarz, Rosa et al. 1998), and a catalytic C-terminal domain (*i.e.* the HECT domain) that is divided into an amino terminal lobe (N lobe) and a carboxyl-terminal lobe (C lobe). In details, the N lobe is about 250 amino acids and contains the E2 binding site; while the C lobe consists of 100 amino acids and contains the active-site cysteine required for the transthiolation reaction (Eletr and Kuhlman 2007). Interestingly, it is known that HECT E3s have a different specificity for UBE2 binding. For example, E6AP binds Ubch7/UBE2L3 and the closely related Ubch8/UBEL6 in a specific manner (Schwarz, Rosa et al. 1998) whereas *Saccharomyces cerevisiae* Rsp5, which share the domain organization with nine human HECT E3 (*i.e.* NEDD4 or Itch/AIPA), binds only Ubch5 and Ubch6 (Schwarz, Rosa et al. 1998). However, an important feature of HECT E3 ligase is that the identity of the E2 did not influence the type of ubiquitin chains formed by HECT E3s. In contrast with E2/RING E3 complex where the identity of the E2 has clearly been shown to determine the type of chains formed (Kim and Huibregtse 2009), the only essential function of the E2 in the HECT model is to transfer ubiquitin to the HECT E3 ligase. It has been demonstrated that different HECT E3s have specificities for the types of polyubiquitin chains that they synthesize. For example, E6AP is highly specific for catalysis of K48-linked polyubiquitylation (Wang and Pickart 2005); human KIAA10 HECT E3 preferentially catalyzes K48 and K29 linkages (Wang, Cheng et al. 2006). In contrast, *Saccharomyces cerevisiae* Rsp5 preferentially synthesizes K63 chains *in vitro* and *in vivo* (Kim and Huibregtse 2009). Recently, Kim and Huibregtse (2009) have demonstrated that the determinants of HECT E3 ligases for chain type specificity are within the last ~60 amino acids of the C lobe.

To date, 28 proteins have been found to belong to HECT E3s family and it is relevant that, in addition to ubiquitylation, they regulate the trafficking of many receptors, channels, transporters and viral proteins. This is the reason for which they play an important role in sporadic and hereditary human diseases including cancer, cardiovascular (Liddle's syndrome) and neurological (Angelman syndrome) disorders (Scheffner and Staub 2007).

2. RING motif-containing E3s

The RING motif was first identified in the early 1990s in the protein encoded by the Really Interesting New Gene 1 (Freemont, Hanson et al. 1991). The canonical RING consensus sequence has been defined as Cys-X₂-Cys-X₉₋₃₉-Cys-X₁₋₃-His-X₂₋₃-Cys/His-X₂-Cys-X₄₋₄₈-Cys-X₂-Cys, where X can be any amino acid residue (Borden 2000). Three-dimensional structures of RING domains revealed that its conserved cysteine (Cys) and histidine (His) residues are buried within the domain's core, where they help maintaining the overall structure through coordination of two atoms of zinc. The RING domain binds two Zn²⁺ ions in a unique "cross-brace" arrangement, which distinguishes it from tandem zinc fingers and other similar motifs (Borden 2000). Unlike zinc fingers, the zinc coordination sites in a RING domain are interleaved, yielding a rigid, globular platform for protein-protein interaction, hence RING domain (Freemont, Hanson et al. 1991). To date, numerous RING variants have been discovered, including two important classes: RING-HC and RING-H2. The classification depends on whether there is a Cys or a His in the fifth of the eight Zn²⁺ coordinating sites. The B-box domain of the TRIM subfamily of RING E3s (see paragraph 1.5) and the U-box domain (see below) are structurally related to the RING. However, whether such consensus sequence variations have functional relevance remains unclear.

RING finger proteins possess ubiquitin ligase activity by themselves or as a part of multisubunit E3s by directly binding ubiquitin-conjugating enzymes (UBE2) (Deshaies 1999; Lorick, Jensen et al. 1999). The precise nature of RING-E2 interaction has been first probed by the NMR analyses of BRCA1 and CNOT4 complexed with UbCH5/UBE2D (Brzovic, Keefe et al. 2003; Dominguez, Bonvin et al. 2004). Moreover, the crystal structure of c-Cbl RING domain bound to UbCH7/UBE2L3 together with mutational analyses highlighted residues on the RING and UBE2 that play a crucial role in sustaining the interface. It is clear that the loop regions comprising the zinc coordination sites of the RING domain and the central helix that connects the first and the second coordination sites together form a cleft on the surface of the RING to which E2s bind (Zheng, Wang et al. 2000). X-ray and NMR data highlighted that Ile383 and Trp408 of c-Cbl and equivalent residues in other RING proteins have been consistently implicated in the interaction with UBE2s. Even if functional studies of the RING E3s typically employ mutations in the zinc-binding residues to inactivate the RING domain, mutation of the Ile and Trp amino acid residues in c-Cbl, CNOT4 and other RING E3s eliminate RING-E2 interaction and E3 ubiquitin activity (Joazeiro, Wing et al. 1999; Albert, Hanzawa et al. 2002)

Interestingly, as already observed for HECT E3/UBE2 interactions (see above), RING E3/UBE2 interactions occur in a specific way. It has been proven that RING E3s that interact with UbCH7/UBE2L3 and the related UbCH8/UBE2L6 cannot interact with UBE2D/E family members (Moynihan, Ardley et al. 1999; Martinez-Noel, Muller et al. 2001). To date, even if many examples are known to sustain this thesis (*i.e.* Cbl/UBE2L3 or CNOT4/UBE2D exclusive binding) there is not enough information about RING amino acid residues assuring specificity to RING E3/E2 interaction.

Many RING finger proteins possess ubiquitin ligase activity by themselves or as a part of multisubunit E3s (Deshaies 1999). Certain members, Mdm2 (Boyd, Tsai et al. 2000; Geyer, Yu et al. 2000), Ubr1/E3 α

(Kwon, Reiss et al. 1998), and Parkin (Shimura, Hattori et al. 2000), for example, are monomers or homodimers and contain both the RING finger domain and the substrate-binding/recognition site in the same molecule. Many others are part of multisubunit complexes; among them are the APC involved in the degradation of cell cycle regulators (Page and Hieter 1999), the von-Hippel-Lindau-Elongins B and C (VBC)-Cul2-RING finger complex (Iwai, Yamanaka et al. 1999; Lisztwan, Imbert et al. 1999) involved in the degradation of HIF1- α (Ivan M et al., 2001) and the Skp1-Cullin/Cdc53-F-box protein (SCF)-RING finger complexes involved in degradation of signal- and cell cycle-induced phosphorylated proteins (De Sepulveda, Ilangumaran et al. 2000).

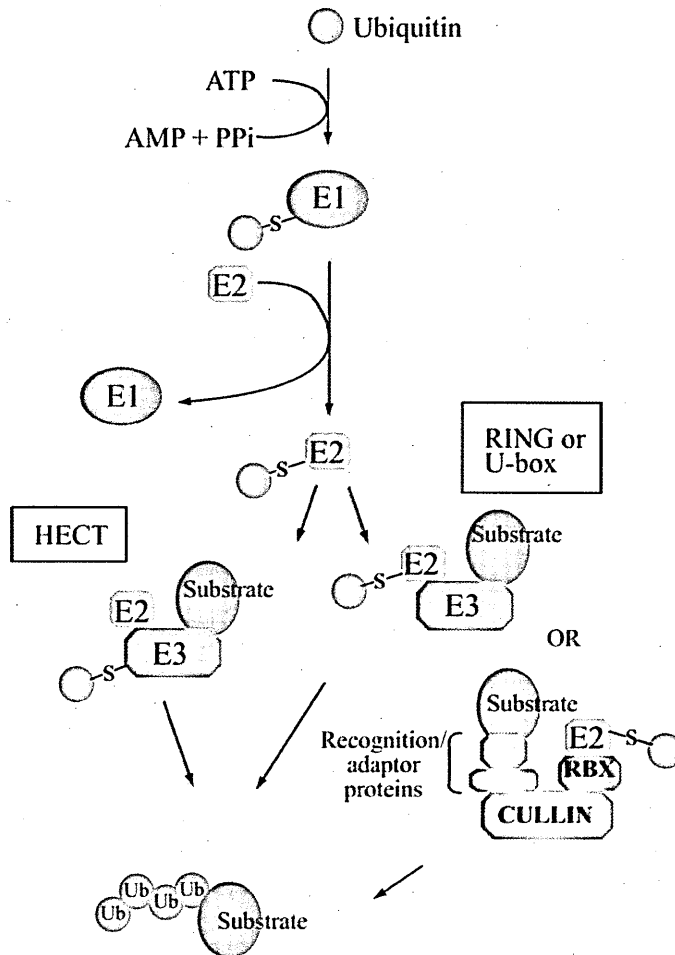
3. E4/U box-containing proteins

E4 defines a protein family that shares a modified version of the RING finger motif. The predicted three-dimensional structure of the U-box is similar to that the RING finger, despite the lack in the former of the hallmark metal-chelating residues present in the latter (Aravind and Koonin 2000). The prototype U-box protein, *Saccharomyces cerevisiae* Ufd2, was originally described as an ubiquitin chain assembly factor (also known as E4) that promotes the polyubiquitylation of artificial fusion proteins in conjugation with E1 (Uba1), E2 (Ubc4) and E3 (Ufd4, a HECT-type E3 enzyme) (Koegl, Hoppe et al. 1999). It has been highlighted that E4 is required for further elongation of an oligoubiquitin chain of certain substrates and the resulting polyubiquitin proteins are then recognized by the 26S proteasome for degradation.

The observation that U-box is a derived version of RING motif suggested that possibility that U-box could also function as E3 enzymes. Indeed, Jiang and coworkers (2001) showed that CHIP, a U-box protein,

functions as an E3 ubiquitin ligase (Jiang, Ballinger et al. 2001). More recently Xu and coworkers (2008) resolved both CHIP/UbcH5 and CHIP/Ubc13 crystal structures, showing that CHIP interaction with these two different enzymes results in the formation of different types of polyubiquitin chains as already observed for the other E3 ligases (Xu, Belunis et al. 2003; Xu, Kohli et al. 2008). Moreover, the E3 ligase activity has been demonstrated for all six mammalian members so far identified (Hatakeyama, Yada et al. 2001). Thus, it seems that U-box proteins possess E3 activity and that their E4 activity likely represents a specialized type of E3 activity apparent with oligoubiquitylated artificial fusion proteins as substrate.

Figure 2



(Dreher and Callis 2007)

A comparison of ubiquitin ligases. HECT domain E3 ligases transiently accept ubiquitin from the UBE2, in a thiolester linkage, before transferring it to the substrate (left branch of the pathway). RING and U- box containing E3 ligases facilitate direct transfer of the ubiquitin from the UBE2 to the substrate (right branch of the pathway). SCF complex (Skp1-Cullin-F box) is a prototype of multisubunit complex RING E3 ligases. Cullins serve as a scaffold to bind a RING finger protein, Roc1/Rbx1, to a specific E2.

1.3 Role of Ubiquitylation

Substrate proteins can be modified by ubiquitin in different ways. A single ubiquitin may be conjugated to a single Lysine (Lys) residue of the substrate (monoubiquitylation) or multiple ubiquitins can be linked via one of the seven Lys residues of the ubiquitin to form short oligoubiquitin chains (2- to 4-ubiquitin moieties) or long polyubiquitin chains (>4-ubiquitin moieties). The chains can be either linear or branched, where two ubiquitin molecules are linked to a single ubiquitin. However the biological significance of this type of linkage is still unclear.

Distinct ubiquitin modifications define different biochemical fates. For example, monoubiquitylation is involved in the DNA repair, endosomal sorting, histone regulation, virus budding and nuclear export; whereas Lys48- and Lys11-linked polyubiquitin chains target proteins for the degradation by the 26S proteasome. Lys63-linked polyubiquitin chain has recently emerged as a novel post-translational modification of remarkable functional interest for its involvement in the DNA repairs and fine-tuning signal transduction pathway. It is known that Lys63-linked polyubiquitin chain creates docking sites for scaffold proteins involved in the regulation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways. These biochemical routes are of great relevance in the response of the immune system against pathogens and in inflammation. Instead, much less is known about the precise function and topology of chains that are linked through Lys6, Lys27, Lys29 and Lys33 and structural analysis are needed to understand if these chains also have peculiar conformational properties (Figure 3).

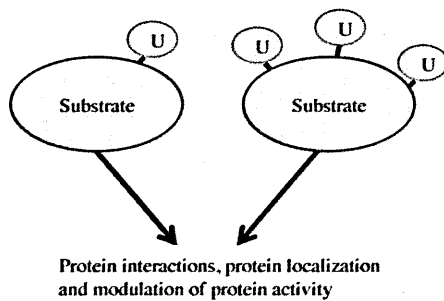
The nature of ubiquitin conjugation by E2/E3 complexes is critical because the outcome of ubiquitylation is generally determined by the topology of the conjugate. In particular, whereas target protein selectivity is provided by the E3, E2 determines the specificity for the lysine residue of the acceptor ubiquitin in polyubiquitin chains formation mediated by RING and

U-box E3, but not by HECT E3s (Kim, Kim et al. 2007). E2s that direct specificity for Lys48-polyubiquitin chains are, for example, human Cdc34 or UBE2K, while the formation of the Lys63-polyubiquitin chains requires a heterodimer composed of Ubc13/UBE2N and either UBE2V1 or UBE2V2 (Thrower, Hoffman et al. 2000). Several examples have been described where a single RING can recruit different E2s that have different linkage specificities. In each case, the output of the reaction is defined by the known specificity of the E2 (Christensen, Brzovic et al. 2007; Kim, Kim et al. 2007).

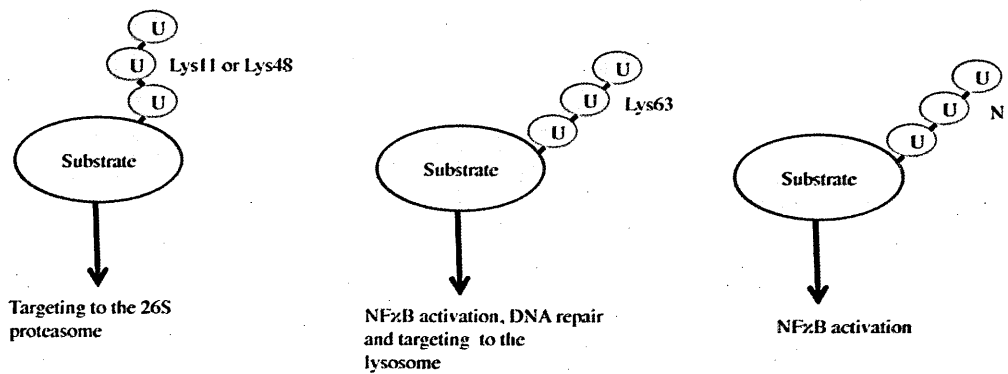
The formation of polyubiquitin chains needs two steps: substrate (mono)ubiquitylation and chain elongation (polyubiquitylation). Monoubiquitylation seems to lack an inherent specificity for a particular lysine residue on the substrate, whereas chain elongation occurs on a particular Lys residue of ubiquitin, for example Lys48 or Lys63. Nevertheless, the details of polyubiquitin chain formation remain elusive (Hochstrasser 2006). A major unanswered question concerns how the decision is made by the ubiquitin machinery as to whether mono- or polyubiquitylate a substrate. One possibility is that different subsets of ubiquitin ligases have specificity for the two different modifications. For example, the ubiquitin ligase Mdm2 mediates monoubiquitylation of p53, whereas p300 has been suggested to promote p53 polyubiquitylation (Grossman, Deato et al. 2003). Alternatively, an individual ubiquitin ligase might mediate either mono- or polyubiquitylation, depending on the nature of the substrate or other molecular specifiers. Recently, a role for ubiquitin-interacting domains (such as the ubiquitin-interacting motif, UIM, or the Cue1-homologous domain, CUE) in the determination of monoubiquitylation of endocytic proteins has been proposed based on the frequent monoubiquitination of proteins containing these domains (Polo, Confalonieri et al. 2003).

Figure 3

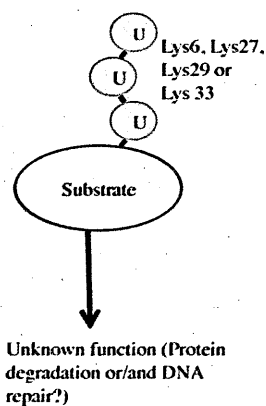
(a) Monoubiquitylation or multi-monoubiquitylation



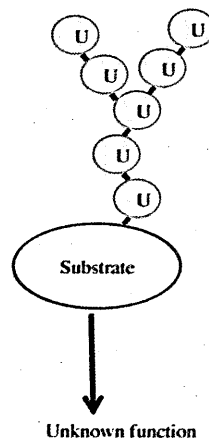
(b) Polyubiquitylation



(c) Polyubiquitination



(d) Branched or forked ubiquitin chains



Examples of different ubiquitin chains. Substrate proteins can be modified with a single ubiquitin (mono-ubiquitylation) or multiple (multi mono-ubiquitylation) sites with different fate. Alternatively, substrates can be modified either with a chain of ubiquitin molecules (polyubiquitylation) linked to one of the seven Lys residues or with an Ubiquitin chains containing branches.

1.3.1 The Ubiquitin-proteasome system.

The Ubiquitin-proteasome system (UPS) leads to the degradation of the proteins by two discrete and successive steps: tagging of the substrate by covalent attachment of multiple ubiquitin molecules and degradation of the tagged protein by the 26S proteasome complex with the release of free and reusable ubiquitin.

The proteasome is a large ATP-dependent proteolytic complex that mediates the degradation of most short-lived proteins that control cell cycle, transcription, DNA repair, apoptosis and other cellular processes. The proteasome is also responsible for the degradation of abnormal or damaged proteins and therefore plays an important role in quality control. The 26S proteasome is a 2.5 MDa protein complex consisting of two subcomplexes: the catalytic 20S core particle (CP or 20S proteasome) and the 19S regulatory particle (RP, also known as 19S proteasome or PA700 (Groll, Ditzel et al. 1997; Nickell, Beck et al. 2009)). The CP is a barrel-shaped structure of a stack of four seven-subunit rings in a $\alpha_7\beta_7\beta_7\alpha_7$ configuration (**Figure 4**). Both exterior rings contain one set of seven different α subunits; and both interior rings contain one set of seven different β subunits. The CP performs three types of catalytic activities inside its chamber: chymotrypsin-like, trypsin-like and caspase-like activities that are provided by β_5 , β_2 and β_1 subunits, respectively (Heinemeyer, Fischer et al. 1997).

Cristal structures of the CP from archaeon, yeast and mammals have been solved (Unno, Mizushima et al. 2002). There is a very narrow pore or gate at the centre of the α subunit ring where protein substrates enter the CP chamber. The gate is closed in a free CP by interactions among the N-termini of the α subunits blocking substrate entry into the proteolytic chamber. Either α subunits can be "capped" by a 19S regulatory subunit that can be further dissected into two multisubunit structures: the "base" that binds directly to the α ring of the 20S core particle, and the "lid" where polyubiquitin is bound (**Figure 4**). The "base" consists of six AAA+ ATPases (Rpt1-6) and three

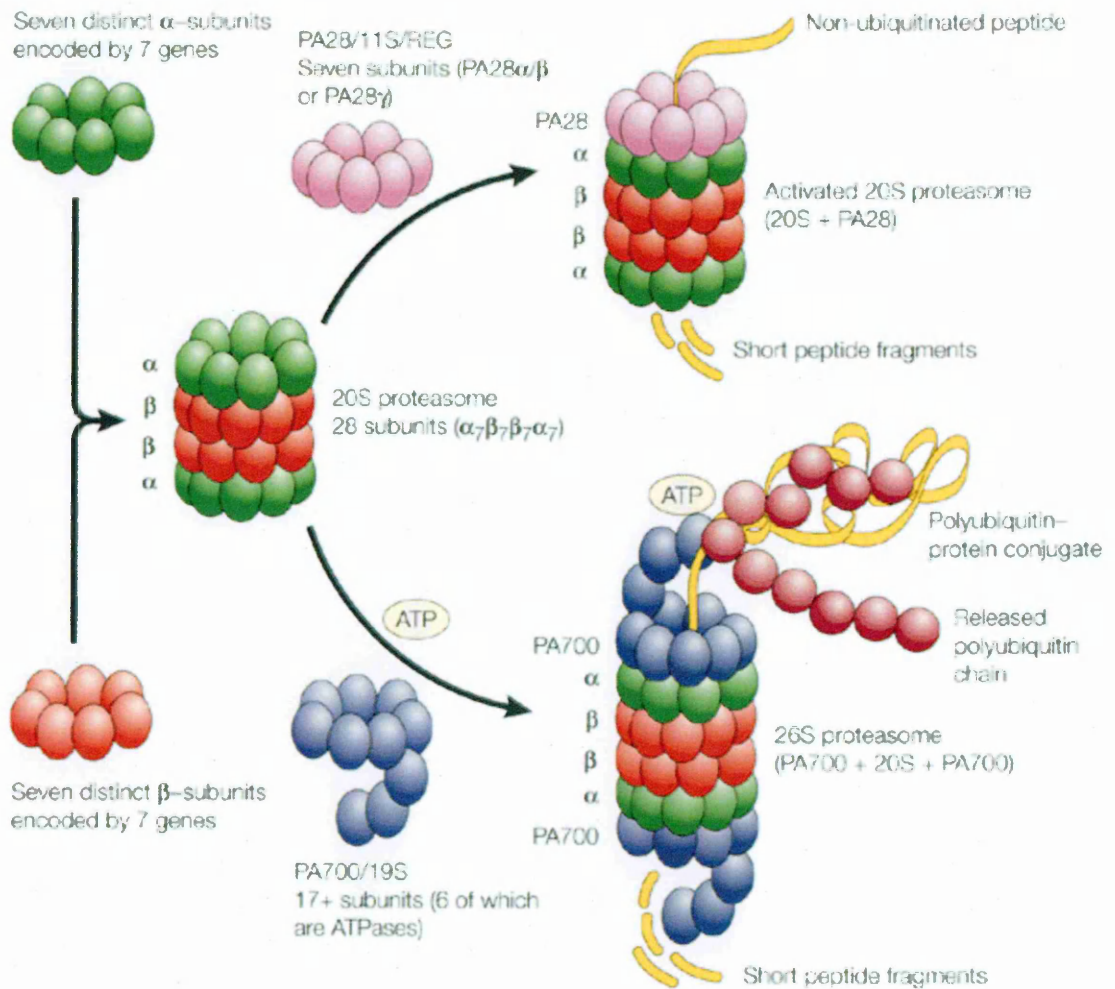
non-ATPase subunits (Rpn1, Rpn2 and Rpn13), whereas the “lid” includes at least nine non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15/Sem1) whose enzymatic activity is still unknown. An alternative form of regulatory subunit called the 11S particle can associate with the core in essentially the same manner as the 19S particle; the 11S may play a role in degradation of foreign peptides such as those produced after infection by a virus (**Figure 4**).

The 19S regulatory particle is responsible for recognizing ubiquitylated proteins and other potential substrates of the proteasome. Then, the 19S regulatory ATPases open the gate in the 20S that blocks the entry of substrates into the degradation. The mechanism by which the proteasomal ATPases open this gate, has been recently clarified by David M. Smith and collaborators (Smith, Chang et al. 2007). The 19S ATPases contain a conserved C-terminal hydrophobic-tyrosine-X motifs (HbYX) that are essential for 19S to associate with the 20S and open its gated-channel for substrate entry. Upon ATP binding, these C-terminal residues bind to pockets between the 20S α -subunits, and tether the ATPase complex to the 20S proteolytic complex thus joining the substrate unfolding equipment with the 20S degradation machinery. Binding of these C-termini into these 20S pockets by themselves stimulates opening of the gate in the 20S much like a "key-in-a-lock" opens a door (Smith, Chang et al. 2007; Rabl, Smith et al. 2008). Also, because the 20S particle's central channel is narrow and gated by the N-terminal tails of the α ring subunits, the substrates must be at least partially unfolded before they enter the core. The passage of the unfolded substrate into the core is called *translocation* and necessarily occurs after deubiquitylation. After degradation of the substrate, short peptides derived from the substrate are released, as well as reusable ubiquitin.

The ubiquitin-mediated proteolysis of a variety of cellular proteins plays an important role in many basic cellular process like cell cycle, differentiation and development, involvement in the cellular response to stress

and extracellular effectors, morphogenesis of neuronal networks, DNA repair, regulation of the immune and inflammatory responses. Moreover, the UPS is intricately involved in protein localization and membrane trafficking (Hicke 2001). The list of cellular proteins that are targeted by ubiquitin is growing rapidly; among these are cyclins, cyclin-dependent kinase inhibitors, tumor suppressors as well as cell surface receptors and endoplasmic reticulum (ER) proteins. Thus, it is not surprising that aberrations in the ubiquitin system have been implicated in the pathogenesis of many inherited and acquired human pathologies as well as neurodegenerative disease (Ciechanover, Orian et al. 2000).

Figure 4



(McNaught, Olanow et al. 2001)

Formation of the 26S proteasome. The 26S proteasome is a multicatalytic protease that is found in the cytosol, perinuclear regions and nucleus of eukaryotic cells. It consists of a 28-subunit catalytic core — 20S proteasome (2,100 kDa) — which is an assembly of two outer and two inner heptameric rings stacked axially to form a hollow cylindrical structure in which proteolysis occurs. The outer rings comprise seven different α-subunits that serve as an anchor for the multisubunit ATPase-containing PA700 regulator that binds to form a complex referred to as the 26S proteasome.

1. ERAD

The UPS was thought initially to degrade only cytosolic proteins. Recent evidence suggest that substrates for ubiquitylation and subsequent degradation are found throughout the cell, in the cytosol, nucleus, ER lumen, and membrane, and cell surface membrane (Bonifacino and Weissman 1998). Normally, ER membrane proteins or proteins that cross the ER membrane are either retained in the ER or traverse the ER lumen to their final destinations: the Golgi apparatus, cell surface membrane, extracellular environment and the lysosomal system. The ER is equipped with a stringent quality control system that monitors the proteins that are synthesized and folded in the ER. This ER quality control system (ERQC) is able to discern between the correctly folded proteins that exit the ER *en route* to their final destinations and the misfolded or unfolded proteins that are retained and refolded in the ER. Proteins that are terminally misfolded are selectively transported from the ER into the cytosol, and subsequently ubiquitylated and degraded by the proteasome, a process called ER-associated degradation (ERAD). During ERAD, misfolded proteins are delivered to 26S proteasome, which resides in the cytoplasm. The destruction of ERAD substrates requires polypeptide recognition, delivery from the ER to the cytoplasm (termed “retrotranslocation or dislocation”) and in most cases ubiquitylation, which ensures efficient delivery to the proteasome. Intense interest has been focused on identification of the protein conduction channels through misfolded proteins can be transported from ER to the cytosol. Early studies suggested that Sec61, the import channel for proteins into the ER, might also be the retrotranslocon through which this dislocation is effected (Plempner, Bohmler et al. 1997). Another such candidate is Derlin-1, a polytopic protein implicated in the targeting of several substrates for degradation (Lilley and Ploegh 2004) (Ye, Shibata et al. 2004) In addition, ER-resident ubiquitin ligases such as Hrd1 have been suggested to form part of this channel (Carvalho, Goder et al. 2006)

In mammalian cells, similar but distinct ERAD components direct the

translocation of misfolded proteins from the ER to the cytosol for degradation via the ubiquitin-proteasome system. Although the degradation pathway of ERAD substrates traverses the “canonical” E1/E2/E3 proteasomal route, it is nevertheless equipped with unique components essential for the retrograde transport. For example, there are two mammalian homologs for each of the yeast enzymes Ubc6 and Ubc7, which are responsible for ERAD in yeast: UBE2J1 and UBE2J2, mammalian homologs of Ubc6, and UBE2G1 and UBE2G2, mammalian homologs of Ubc7. The formers have hydrophobic sequences at their C termini that mediate post-translational insertion into the ER membrane; the latter are cytosolic proteins. To date, there is little evidence implicating UBE2G1 in ERAD; instead, UBE2G2 is strongly implicated in ERAD, functioning with multiple ERAD E3s (Kikkert, Doolman et al. 2004). More evidences also support roles for UBE2J1 and UBE2J2 (Lenk, Yu et al. 2002; Arteaga, Wang et al. 2006).

The first mammalian ubiquitin ligases integral to the ER membrane shown to function in ERAD were gp78 (Fang, Ferrone et al. 2001) and Hrd1 (Kikkert, Doolman et al. 2004). In addition, some cytosolic E3s also function in ERAD like Parkin (Imai, Soda et al. 2001) and CHIP (Younger, Chen et al. 2006). Finally, it has to been underscored that the accumulation of ERAD substrates may induce the unfolded protein response (UPR), which if unresolved will trigger either apoptosis or autophagy (Ding, Ni et al. 2007).

1.3.2 Monoubiquitylation

Monoubiquitylation is implicated in a plethora of cellular processes such as endocytosis of plasma membrane proteins, DNA repair, histone activity and transcriptional regulation.

Histones were found to be monoubiquitylated more than 20 years ago (Haas, Bright et al. 1988), but only during the past years several functions have been identified for histone ubiquitylation. Histones H2A and H2B are modified by monoubiquitin or short ubiquitin chains on lysines (Lys) in their carboxy-terminal tails. In mammalian cells, ~10% of H2A and ~1% of H2B are ubiquitylated but in the budding yeast *Saccharomyces cerevisiae*, H2B is the predominant ubiquitylated histone. Further evidence that is consistent with a role for histone ubiquitylation in meiosis comes from the examination of mutants that are defective in the Rad6 (E2), which catalyses the ubiquitylation of H2A and H2B in yeast. Yeast *rad6* mutants, like the H2B mutants, cannot sporulate (Prakash 1989) and mouse knockouts lacking a Rad6 homologue (HR6B) are specifically defective in spermatogenesis, leading to male infertility (Roest, van Klaveren et al. 1996).

In mammalian cells, several ion channels and signal-transducing receptors that undergo regulated internalization are ubiquitylated in response to an extracellular signal and ubiquitylation regulates their endocytic transport. The first clue that monoubiquitin, and not ubiquitin chains, controls these processes came from studies on Ste2p, a pheromone G protein-coupled receptor in *Saccharomyces cerevisiae* (Terrell, Shih et al. 1998). More recent studies in mammalian cells have shown that chimerae consisting of monoubiquitin fused to the cytoplasmic regions of the invariant chain of the interleukin-2 receptor a chain or epidermal growth factor receptor (EGFR) can be efficiently downregulated by monoubiquitylation. These receptors are constitutively internalized from the cell surface and targeted to the late endosomal/lysosomal compartment, indicating that a single ubiquitin carries both internalization and sorting signals. Monoubiquitin is not only required as

an internalization signal on endocytic cargo; it might also control the activity of the endocytic machinery. EPS15, a protein that interacts with the Clathrin-based endocytic machinery, becomes monoubiquitylated upon stimulation of cells with EGF, a ligand that upregulates activity of the endocytic machinery. The most important E3 ligases that are known to modify plasma membrane proteins are: Cbl and Nedd4. Cbl is a proto-oncoprotein that recognizes and ubiquitylates activated, phosphorylated growth factor receptors by binding to phospho-tyrosines. It is required to downregulate activated receptors by endocytosis and subsequent degradation in the lysosome. Nedd4 binds to and ubiquitylates the epithelial sodium channel that undergoes ubiquitin-dependent degradation in the lysosome. PPXY motifs in channel subunits are required for Nedd4 interaction. The yeast homologue of Nedd4, Rsp5, is required for the ubiquitylation and internalization of several proteins.

It is well documented the relationship between monoubiquitylation and Fanconi Anemia (FA) in which patients are highly sensitive to DNA cross-linking agents such as mitomycin C (Smogorzewska, Matsuoka et al. 2007). Eight proteins form the Fanconi anemia complex, a nuclear E3 ubiquitin ligase which monoubiquitylates Fanconi anemia group D2 protein (FANCD2) and its paralog, Fanconi anemia complementation group I protein (FANCI), both of which are involved in the FA DNA repair pathway (Smogorzewska, Matsuoka et al. 2007). FANCD2 and FANCI form an “ID complex” that co-localizes to chromatin upon DNA damage. Following DNA damage, FANCD2 is phosphorylated by the S phase checkpoint kinase, CHK1, which triggers DNA damage inducible monoubiquitylation of FANCD2 (Zhi, Wilson et al. 2009). FANCI is subsequently monoubiquitylated, DNA damage induced foci are formed and additional proteins involved in DNA damage response are recruited. Interestingly, FANCD2 and FANCI demonstrate a “dual ubiquitin-locking mechanism” where maintenance of monoubiquitylation of either protein is dependent on the monoubiquitylation status of the other (Smogorzewska, Matsuoka et al. 2007). Thus

phosphorylation and monoubiquitylation of the ID complex is required for recruitment to the damage site on chromatin where it directs the removal of damaged DNA and DNA repair (Smogorzewska, Matsuoka et al. 2007). Recently, Shereda and co-workers have identified FAN1, a protein with a nuclease domain and a ubiquitin-binding domain (UBZ4), which localizes to stalled replication forks and is required for mitomycin C resistance (Shereda, Machida et al. 2010). They demonstrated that the UBZ4 domain binds directly to ubiquitin and is required for proper localization of FAN1 upon DNA damage. It was thought that FAN1 is recruited to chromatin by binding to monoubiquitylated FANCD2, and its recruitment and nuclease activity are required for DNA interstrand crosslinks (ICL) repair.

1.4 Ubiquitin-like modification

To date, eleven small peptides have been identified as ubiquitin-like proteins (UBLs) even though some show very little resemblance to ubiquitin (Schwartz and Hochstrasser 2003) (**TABLE 1**). UBLs attachments principally regulate interactions with other macromolecules, such as proteasome-substrate binding or recruitment of proteins to chromatin. Different UBL systems use related enzymes to attach specific UBLs to proteins (or other molecules), and most UBL attachments are transient. Although some of the biological functions of these modifications are starting to be deciphered, in most cases we have yet to learn how the UBL modification elicits a particular change in protein activity. Moreover, the ubiquitin family of protein modifiers is thought to derive from a single ancestral conjugation system (Schwartz and Hochstrasser 2003). This may raise the question of whether, after their multiplication and divergence during evolution, some of the UBL conjugation systems retain any functional overlap or cross-regulation.

Attachment of ubiquitin-like proteins (UBLs) to their targets via multienzyme cascades constitutes a central mechanism through which protein functions are modulated. All UBLs display the common ubiquitin superfold, and in general, have their own discrete E1-E2-E3 cascades, and impart distinct functions to their targets. As in the ubiquitylation pathway, UBL modification is initiated by their dedicated family of mechanistically and structurally related E1 enzymes which are essential for all further conjugation (Huang, Hunt et al. 2007). E1s play a critical function in initiating UBL conjugation cascades: selecting the correct UBLs for the pathway and transferring the UBL to their cognate E2.

Besides the well-studied SUMO (see below), a number of other UBLs function in diverse biological pathways. The conjugation of two UBLs – interferon stimulated gene 15 (ISG15) and FAT10 - are under the control of the interferon system, which responds to viral signals. ISG15 – the product of

an interferon inducible gene - is activated by the E1 UBA7 and is transferred to dozens of targets in a wide range of pathways through a specific E2, ubiquitin conjugating enzyme 8 (UBCH8), whose expression is also under interferon control (Zhao, Beaudenon et al. 2004; Zhao and Blobel 2005). Additional UBLs, including the autophagy related protein 8 (ATG8) and ATG12 families, ubiquitin fold modifier 1 (UFM1) and ubiquitin-related modifier 1 (URM1) are activated by their own E1 enzymes (ATG7, UBA5 and UBA4, respectively). ATG8 and ATG12 are involved in multiple steps in autophagy, a process by which cells degrade their cytoplasmic organelles through the lysosome. In contrast, URM1 is functionally distinct in that it is used in biosynthetic reactions that involve sulfur transfer. Another UBL is NEDD8, which is an 81 amino acids ubiquitin-like protein (Liakopoulos, Doenges et al. 1998). Neddylation is initiated by a specific E1 enzyme, NAE1-UBA3 (Gong and Yeh 1999) that first uses ATP to form a NEDD8 adenylate and then transfers NEDD8 from the adenyl group to a specific cysteine (Cys) within NAE forming an "activated" NAE-NEDD8 thioester. The activated NEDD8 is then transferred to the active site cysteine in either UBE2M or UBE2F, the E2s specific for the NEDD8 pathway. Finally, the resulting UBE2~NEDD8 thioester conjugate serves as the direct source of NEDD8 to be covalently attached to a cullin's acceptor lysine (Lys). Recent studies suggest that SCCRO (DCN1), a protein that has been shown to interact with UBC12 and cullins, acts as a scaffold-type E3 ligase for cullin neddylation (Kurz, Chou et al. 2008). NEDD8 is then removed from the cullins by the isopeptidase activity of the metalloprotease CSN5/JAB1 subunit of the COP9 signalosome (Wei 2009). The modification of ubiquitin E3s by UBL is also involved in cross-regulation pathway. For instance, both SUMO and ubiquitin can modify the same residues of MEK1 and PCNA. SUMOylation can protect a protein from degradation by preventing ubiquitin ligation (Muller, Hoege et al. 2001) or it can prevent some other effect of

ubiquitin ligation, such as the stimulation of DNA repair in the case of PCNA (Hoegel, Pfander et al. 2002).

1. SUMOylation pathway

The ubiquitin-like protein SUMO is distantly related to ubiquitin (16-18% identity) and was first identified in mammals, where it was found to be covalently linked to the GTPase activating protein RanGAP1 (Mahajan, Delphin et al. 1997). The E1 activating and E2 conjugating enzymes involved in SUMOylation are highly related to the E1 and E2 enzymes that participate in ubiquitylation. In contrast to the ubiquitin system where dozens of E2 enzymes have been identified, Ubc9 is the only known SUMO E2 conjugating enzyme. All SUMO isoforms are conjugated via a conserved enzymatic cascade that resembles that of ubiquitin conjugation (**Figure 5**). SUMO is first activated by formation of a thioester bond between its C-terminal glycine (Gly) and the catalytic cysteine (Cys) of the heterodimeric E1 activating enzyme (Aos1/Uba2, also named SAE1/SAE2). This step requires ATP hydrolysis. SUMO is then transferred to the catalytic Cys of the single E2 conjugating enzyme Ubc9. The last step consists in the transfer of SUMO from the E2 to the ϵ -amino group of a lysine (Lys) side chain on the substrate, which results in isopeptide bond formation. For this, Ubc9 needs to recognize a specific acceptor site in the target. Many – but not all – targets contain the so-called SUMO consensus motif, Ψ KxE, where Ψ is a large hydrophobic residue and K the acceptor lysine. The interaction between Ubc9 and most targets is not stable enough for efficient transfer, and therefore requires additional proteins, the so-called E3 ligases. Currently known ligases include PIAS (Protein inhibitor of activated STAT) proteins (Shuai and Liu 2005), the nucleoporin RanBP2/Nup358 (Pichler, Gast et al. 2002) and the polycomb protein Pc2 (Kagey, Melhuish et al. 2003). Notably, the different SUMO E3 ligases identified to date have distinct subcellular localizations:

RanBP2 is associated with the nuclear pore complex, the PIAS proteins are found in the nucleoplasm and nuclear bodies, and Pc2 is found in a subnuclear structure called Polycomb body (Kagey, Melhuish et al. 2003). Localization of the SUMO E3 ligases is likely to contribute to functional specificity in vivo. However, the list of SUMO E3 ligases is growing as additional proteins with SUMO E3 ligase activity have been recently identified. For example, histone deacetylase 4 (HDAC4) can promote the SUMOylation of MEF2, independent of its deacetylase activity (Zhao and Blobel 2005). The human topoisomerase I- and p53-binding protein Topors can function as both a ubiquitin and a SUMO E3 ligase for p53 (Weger, Hammer et al. 2003). SUMO conjugation is reversible, and the SUMO-specific protease (SENP) family is responsible for rapid removal of SUMO from SUMOylated protein substrates (Hay 2007). Whether other types of SUMO isopeptidases exist is still not clear. All SENPs share a conserved catalytic domain but have distinct N-terminal extensions, which might be responsible for their different intracellular localisations.

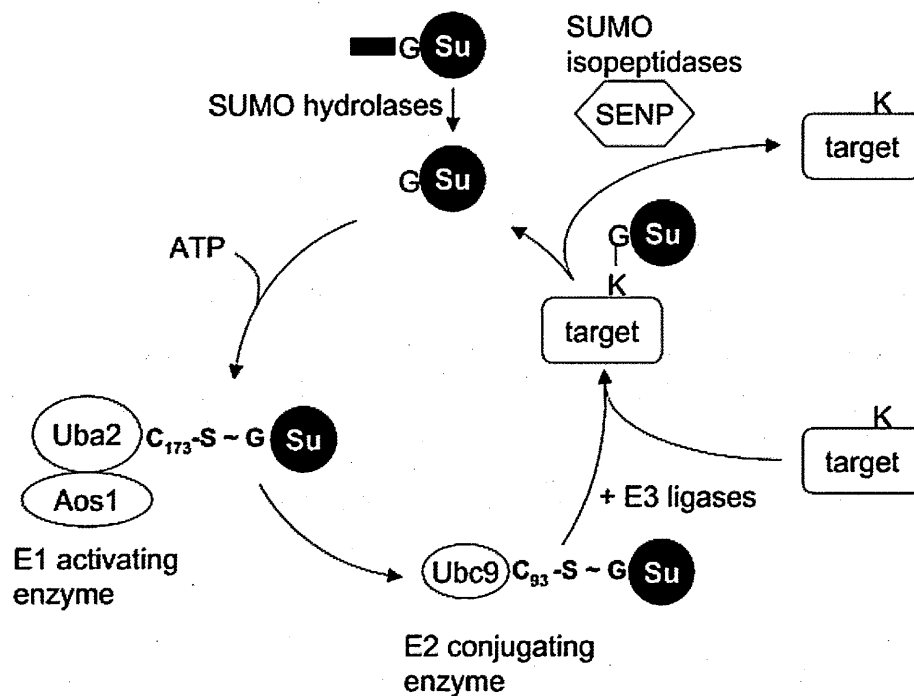
Since the identification of the first SUMO-modified protein, RanGAP, in 1996 (Matunis, Coutavas et al. 1996), a large number of proteins have been shown to be post-translationally modified by SUMO and new substrates of SUMO-modification continue to be identified at a rapid pace. Many of the known targets of SUMOylation are nuclear proteins with important roles in regulating transcription, chromatin structure, and DNA repair. Furthermore, the nuclear targets of many signalling pathways including TGF β , Wnt, and cytokines are post-translationally modified by SUMO. It is interesting to note that some of the proteins that have been found to be modified by SUMO are also post-translationally modified by ubiquitin, often with different consequences (Hunter and Sun 2008). In some cases, SUMO modification has been shown to compete with ubiquitylation or acetylation for common lysine residues. In most cases SUMO modification is likely to regulate protein-protein interactions.

TABLE 1

UBL	Ubiquitin Sequence Homology (%)	E1 and E2 enzymes	Substrates	Functions
ISG15 (UCRP) (2 ubiquitins)	29, 27	E1: UBE1L; E2: UBCH8	PLC γ 1, JAK1, STAT1, ERK1/2, serpin 2a	Regulator of IFN-related immune response
FUB1 (MNSF β)	37	NA	TCR- α -like protein, Bcl-G	Negative regulator of leukocyte activation and proliferation
NEDD8 (Rub1)	58	E1: APPBP1-UBA3; E2: UBC12;	cullins, p53, Mdm2, synphilin-1	Positive regulator of ubiquitin E3s; proteasomal degradation
FAT10 (2 ubiquitins)	29, 36	NA	MAD2	Cell cycle checkpoint for spindle assembly; proteasomal degradation
SUMO1-3	16-18	E1: SAE1/2; E2: UBC9	c-Jun, I κ B α , p53, Mdm2, SOD-1, NEMO, PML, Sam68, RanGAP1, etc.	Transcription regulation, cell cycle progression
Atg 8	10	E1: Apg7; E2: Apg3;	Phosphatidylethanolamine	Autophagy,
Atg 12	17	E1: Apg7; E2: Apg10	Atg 5	Autophagy,
Urm1	12	E1: Uba4	Ahp1	Potential role in oxidative stress response
UBL5 (Hub1)	25	NA	CLK4, Snu66, Sph1, Hbt1	Pre-mRNA splicing, appetite regulation
Ufm1	16	E1: Uba5; E2: Ufc1	NA	Potential role in endoplasmic stress response

Ubiquitin-like proteins for which there is experimental evidence for ligation to other molecules. The ubiquitin superfamily consists of numerous proteins that display structural similarity to ubiquitin and are involved in a range of biological activities.

Figure 5



(Bossis and Melchior 2006)

The SUMOylation pathway. SUMO is first activated in an ATP-dependent process by forming a thioester bond with the catalytic Cys of the E1-activating enzyme, which is a heterodimer consisting of two proteins SAE1 and SAE2 (also known as Aos1 and Uba2, respectively). SUMO is transferred to the catalytic Cys of the E2-conjugating enzyme Ubc9, which directly binds to substrates. Subsequently, SUMO is conjugated to the protein substrate by forming an isopeptide bond between SUMO and the ϵ -amino group of a lysine side chain on the substrate. SUMO conjugation is reversible, and the SUMO-specific protease (SEN) family is responsible for rapid removal of SUMO from SUMOylated protein substrates.

1.5 The TRIM protein family

The proteins that belong to the tripartite motif (TRIM) family (also known as the RBCC family) are defined by the presence of a RING (R) domain, one or two B-box domains and a coiled-coil (CC) region (Borden 2000; Reymond, Meroni et al. 2001) (see below). The RING domain is present in hundreds of other proteins, while the zinc-binding B-box domain is a critical determinant of the tripartite motif family. This motif is invariably present at the N-terminal portion of these proteins, while their C-terminus presents various domains.

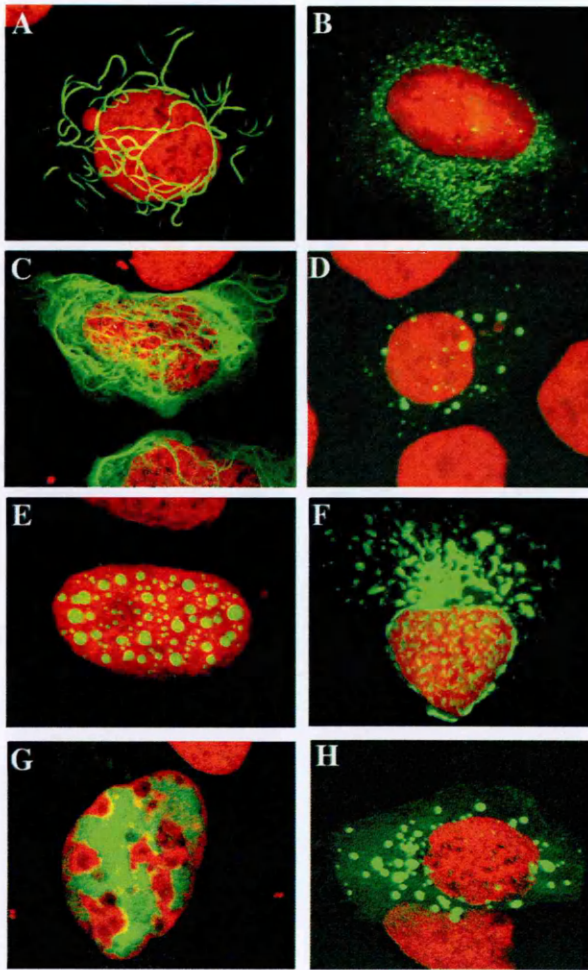
The TRIM proteins self-associate, mainly through their coiled-coil region, and homo-interaction results in the formation of large protein complexes (Reymond, Meroni et al. 2001). 68 proteins belong to this family and are involved in many cellular processes such as apoptosis, cell cycle regulation, and viral response (Sardiello, Cairo et al. 2008).

Many TRIM proteins have been characterized for their subcellular localization and shown to be associated with specific compartments, such as nuclear bodies (PML/TRIM19, TIF1/TRIM24 and RFP/ TRIM27) or the microtubules (MID1/TRIM18 and MID2/TRIM1) (Dyck, Maul et al. 1994; Le Douarin, Zechel et al. 1995; Cao, Duprez et al. 1998; Buchner, Montini et al. 1999; Cainarca, Messali et al. 1999). The great majority of TRIM proteins localize to discrete cytoplasmic or nuclear compartments sometimes associated with a diffusely stained background (**Figure 6**) (Reymond, Meroni et al. 2001). In the case of cytoplasmic TRIM proteins, the cellular compartments are associated with filaments, or assume a cytoplasmic ribbon-like structure (Reymond, Meroni et al. 2001). Other TRIM proteins are concentrated in cytoplasmic bodies of variable size, occasionally located around the nucleus (Reymond, Meroni et al. 2001). Nuclear TRIM proteins localize to structures best described as nuclear body or nuclear sticks. In particular, PML is required for NB formation and is necessary for the

recruitment of other components into the NBs. In fact, deletion of PML results in a lack of formation of NBs (Melnick and Licht 1999).

Genetic alteration in genes encoding TRIM proteins can result in human diseases: TRIM18 is mutated in X-linked Opitz/GBBB syndrome (Quaderi, Schweiger et al. 1997); TRIM19/PML, and TRIM27/RFP, acquire oncogenic activity when fused to RAR α and RET respectively (Takahashi 1988); EFP/TRIM25 is implicated in tumour progression and growth (Urano, Saito et al. 2002); TRIM54 is upregulated in a model of muscle atrophy (Bodine, Latres et al. 2001); TRIM32 is associated with both skin carcinogenesis and Limb-Girdle Muscular Dystrophy type 2H (LGMD2H) (Frosk, Weiler et al. 2002); TRIM37/MUL is involved in the pathogenesis of Mulibrey nanism, a syndromic form that affects muscle, liver, brain and eye (Avela, Lipsanen-Nyman et al. 2000); TRIM20/Pyrin/Marenostrin is mutated in Familial Mediterranean Fever, an inflammatory disease (1997; Chae, Komarow et al. 2003). Recently TRIM protein antiviral activity is emerging (Nisole, Stoye et al. 2005; Ozato, Shin et al. 2008). TRIM5 α was shown to inhibit the replication of lentiviruses including HIV-1 (Stremlau, Owens et al. 2004); TRIM1 and TRIM22 interfere with the replication of N-tropic murine leukemia virus (N-LMV) and human immunodeficiency virus (HIV-1), respectively (Ozato, Shin et al. 2008). TRIM30 α negatively regulates Toll-like receptor (TLR)-mediated NF- κ B activation (Shi, Deng et al. 2008). TRIM21/Ro52 is a target autoantigen in several systemic autoimmune diseases, including Systemic lupus erythematosus (SLE) and Sjögren's syndrome (Hennig, Bresell et al. 2008).

Figure 6



TRIM proteins homomultimerize and associate with specific subcellular structures. Subcellular localization of TRIM29 (A), TRIM4 (B), TRIM2 (C), TRIM5 (D), TRIM8 (E), TRIM13 (F), TRIM28 (G) and TRIM9 (H) (Reymond et al., 2001).

1.5.1 TRIM domain structure

1. The Tripartite motif

As already discussed in the previous paragraph, the RING motif is defined by a regular arrangement of cysteine (Cys) and histidine (His) residues that coordinate two atoms of zinc (**Figure 7**). There are two main RING subtype, H2 and C2. Of the two RING subtypes, the C2, which is characterized by a Cys residue in the fifth coordination site, is found in the TRIM family (Meroni and Diez-Roux 2005). With few exceptions, the RING domain is typically found within 10-20 amino acids of the TRIM protein first methionine (Torok and Etkin 2001).

The B-box domain is another zinc-binding motif that occurs in two flavours, B-box1 and B-box2, which present a similar but distinct pattern of cysteine and histidine residues (**Figure 7**) (Reymond, Meroni et al. 2001). The B-box domains usually adopt a $\beta\beta\alpha$ conformation similar to RING domain's one. B-box1 and B-box2 diverge in the second potential coordination residue, which is a Cys in B-box1 and a His in B-box2. In addition, the B1 and B2 domains have different lengths and, when found together, B-box type 1 always precedes type 2. In TRIM proteins, B-box domain usually mediates the interaction with TRIM's interactor protein.

A coiled-coil region always follows the B-box2 in the entire set of TRIM proteins. This region is approximately 100-residue-long, and it is frequently spilt into two separate coiled-coil motifs. The coiled-coil region in the TRIM family is mainly involved in homo-interaction and in promoting the formation of high molecular weight complexes. Disruption of the TRIM coiled-coil region is associated with diffuse localization (Reymond, Meroni et al. 2001); in contrast, independent deletions of RING or B-box1 and B-box2 induce relocalization of the mutant protein to aberrant cellular structures (Reymond, Meroni et al. 2001).

The striking structural feature of this family is the rigid conserved pattern, combination and order of the domains, which strongly suggests that this minimal structure was selectively maintained to carry out a specialized basic function common to all tripartite motif proteins. The order of the domains within the tripartite motif (RING, B-box1, B-box2, and coiled-coil) is maintained from the N-terminus to the C-terminus, if one domain is absent, the order of the remaining ones is conserved. These observations suggest that the tripartite motif is an integrated functional structure, rather than a collection of separate modules.

2. The C-terminal domain

While the tripartite motif, and especially the B-box domain, is restricted to this protein family, the C-terminal domains found in the TRIM proteins is also present in otherwise unrelated proteins. A number of TRIM proteins do not possess a defined C-terminal domain; in this case, either their coding region is limited to the tripartite motif or the C-terminal portion is not similar to any known domains or proteins. Two thirds of the TRIM members have a B30.2 or PRY-SPRY domain, also known as RFP-like domain having been first identified in TRIM27/RFP (Meroni and Diez-Roux 2005) (**Figure 8**). It is a 170-residue long domain composed of three blocks named after the more conserved amino acid stretches, LPD (also known as PRY), WEVE and LDYE (also known as SPRY domain) motifs (Henry, Mather et al. 1998). Recently RFP region has been associated to TRIM22 formation of distinct nuclear bodies (Sivaramakrishnan, Sun et al. 2009) and TRIM5 protein retroviral restriction (Stremlau, Owens et al. 2004). Interestingly, TRIM20 mutations in RFP domain have been strictly associated to Familial Mediterranean Fever (FMF)(Chae, Komarow et al. 2003). A less frequent C-terminal domain within the TRIM family is the NHL domain. It consists of 2-6 repeats, usually 5 or 6 in the TRIM proteins, of an approximately 40-residue sequence that resembles the WD repeat and that assembles to form multiblade

propeller structure (Slack and Ruvkun 1998). The TRIM proteins that contain a PHD associated to a BROMO domain represent a more homogeneous subfamily composed of four members, the TIF1 proteins, which share high homology along the entire length of their sequence and participate in similar cellular processes (Moosmann, Georgiev et al. 1996).

Figure 7

RING consensus

C-x₂-C-x₁₁₋₁₆-C-x-H-x₂-C-x₂-C-x₇₋₇₄-C-x₂- [CD]

B-box1 consensus

C-x₂-C-x₇₋₁₂-C-x₂-C-x₄-C-x₂- [CH]-x₃₋₄-H-x₄₋₉-H

B-box2 consensus

C-x₂-H-x₇₋₉-C-x₂- [CDHE]-x₄-C-x₂-C-x₃₋₆-H-x₂₋₄- [CH]

RING, B-box1 and B-box2 consensus sequences. Consensus sequences for the RING, B-box1 and B-box2 domains within the tripartite motif RING consensus: in blue are the cysteines involved in the first zinc atom coordination while the red residues are involved in the second metal binding. B-box2 consensus: in blue are the residues involved in zinc coordination.

Figure 8

TRIM 1	R-B1-B2-CC-FN3-SPRY
TRIM 2	R-B2-CC-IGFLMN-NHL(6)
TRIM 3	R-B2-CC-IGFLMN-NHL(6)
TRIM 4	R-B2-CC-SPRY
TRIM 5	R-B2-CC-SPRY
TRIM 6	R-B2-CC-PRY-SPRY
TRIM 7	R-B2-CC
TRIM 8	R-B1-B2-CC-nd
TRIM 9	R-B1-B2-CC-FN3-SPRY
TRIM 10	R-B2-CC-PRY-SPRY
TRIM 11	R-B2-CC-PRY-SPRY
TRIM 13	R-B2-cc-TM
TRIM 14	B2-cc-PRY
TRIM 15	R-B2-CC-PRY-SPRY
TRIM 16	B1-B2-CC-PRY-SPRY
TRIM 17	R-B2-CC-PRY-SPRY
TRIM 18	R-B1-B2-CC-FN3-PRY-SPRY
TRIM 19	R-B1-B2-cc-nd
TRIM 20	PAAD-B2-CC-PRY-SPRY
TRIM 21	R-B2-CC-PRY-SPRY
TRIM 22	R-B2-CC-PRY
TRIM 23	R-B2-CC-ARF
TRIM 24	R-B1-B2-CC-PHD-BROMO
TRIM 25	R-B1-B2-CC-PRY-SPRY
TRIM 26	R-B2-CC-PRY-SPRY
TRIM 27	R-B2-CC-PRY-SPRY
TRIM 28	R-B1-B2-CC-PHD-BROMO
TRIM 29	B1-B2-CC-nd
TRIM 31	R-B2-CC-nd
TRIM 32	R-B1-CC-NHL(5)
TRIM 33	R-B1-B2-CC-PHD-BROMO
TRIM 34	R-B2-CC-SPRY
TRIM 35	R-B2-CC-PRY-SPRY
TRIM 36	R-B1-B2-cc-FN3-SPRY
TRIM 37	R-B2-CC-MATH

TRIM 38	R-B2-CC-PRY-SPRY
TRIM 39	R-B2-CC-PRY-SPRY
TRIM 40	R-B2-CC
TRIM 41	R-B2-CC-PRY-SPRY
TRIM 42	R-B1-B2-cc-FN3
TRIM 43	R-B2-CC-SPRY
TRIM 44	B1-B2-CC
TRIM 45	R-B1-B2-CC-IG FLMN
TRIM 46	R-B1-B2-CC-FN3
TRIM 47	R-B1-B2-cc-PRY-spry
TRIM 48	R-B2-cc
TRIM 49	R-B2-cc-spry
TRIM 50	R-B2-CC-PRY-SPRY
TRIM 73	R-B2-CC
TRIM 74	R-B2-CC
TRIM 52	R-B2
TRIM 54	R-B2-CC-nd
TRIM 55	R-B2-CC-nd
TRIM 56	R-B1-B2-CC-nd
TRIM 58	R-B2-CC-PRY-SPRY
TRIM 59	R-B2-cc-TM
TRIM 60	R-B2-CC-PRY-SPRY
TRIM 61	R-B2-CC-PRY-SPRY
TRIM 62	R-B2-CC-PRY-SPRY
TRIM 63	R-B2-CC-nd
TRIM 64	R-B2-CC-SPRY
TRIM 65	R-B2-CC-PRY-SPRY
TRIM 66	B1-B2-CC-RPT
TRIM 67	R-B1-B2-CC-FN3-SPRY
TRIM 68	R-B2-CC-PRY-SPRY

A schematic representation of the TRIM proteins identified. Colour coding is as follows: red R, RING domain; light blue B1, B-box type1 domain; dark blue B2, B-box type 2 domain; green CC, coiled-coil region; yellow PRY/SPRY, RFP –like region; black PHD-BROMO domain.

1.5.2 TRIM proteins as E3 Ubiquitin Ligases

As reported above, RING finger proteins are the largest class of Ubiquitin Ligases. The broad use of the RING domain in ubiquitylation may suggest that the TRIM family, characterized by the presence of a RING finger within the tripartite motif, represents a subclass of single protein RING finger E3s. This hypothesis is supported by recent experimental data, which demonstrate the E3 ubiquitin ligase activity of some family members.

Efp/TRIM25, an estrogen-responsive gene, has been shown to selectively control the protein level of 14-3-3 σ , a cell cycle regulator (Urano, Saito et al. 2002). This control is exerted, in a proteasome-dependent manner, through direct binding between Efp and both substrate, 14-3-3 σ , and an E2 Conjugating Enzyme, UbcH8 (Urano, Saito et al. 2002). As expected, the E2 enzyme binds the RING domain of Efp while the B-boxes and the coiled-coil region mediate substrate interaction. Mid1/TRIM18, the protein altered in Opitz syndrome, controls the level of the catalytic subunit of microtubular phosphatase 2A (PP2Ac) (Troddenbacher, Suckow et al. 2001). In this case, TRIM18 binds directly to a subunit of the PP2A complex, alpha 4, through the B-box 1 region (Liu, Prickett et al. 2001). TRIM8/GERP regulates SOCS-1 (suppressor of cytokine signalling 1) activity by controlling its degradation through direct binding via its B-box and coiled-coil region (Toniato, Chen et al. 2002). Along the same line, TRIM11 binds and regulates the level of humanin, a neuroprotective 24-residue-peptide, via the coiled-coil SPRY domain (Niikura, Hashimoto et al. 2003) and it is also implicated in the degradation of ARC105 (Ishikawa, Tachikawa et al. 2006). Recent data suggest that TRIM32 interacts with and promotes the ubiquitylation and degradation of PIAS family members (Albor, El-Hizawi et al. 2006), Abl-interactor 2 (Kano, Miyajima et al. 2008) and dysbindin (Locke, Tinsley et al. 2009). The importance of RING domain is due not only to its capacity to recruit the E2, but also to its catalytic role. The preference for specific E2s has been demonstrated for some of the above-mentioned TRIM E3s: UbcH8

but not UbcH7 for Efp (Urano, Saito et al. 2002), UbcH5B but not Ubc8 and Ubc13 for TRIM5 δ (Xu, Yang et al. 2003), UbcH5 and UbcH6 for TRIM32 (Albor, El-Hizawi et al. 2006). Moreover, experimental evidence confirmed that the RING domain is involved in mediating their E3 activity. The central role of this domain is also demonstrated by the plethora of TRIM proteins whose cellular activity is compromised in RING domain mutants.

While the tripartite motif might serve as the module to bring the ubiquitin-E2, bound to the RING domain, in close proximity to the substrate, bound to the B-box/coiled-coil region, the role of C-terminal domain in the E3 activity is still unclear. These domains are also present in non-TRIM proteins and, in some cases, have been shown to interact with proteins involved in the ubiquitylation process. In particular, the B30.2 domain has been reported to interact with proteasome subunits and ubiquitin itself (Suzumori, Burns et al. 2003). It is interesting to notice that another TRIM C-terminal domain, PHD, has been also associated to ubiquitylation (Lu, Xu et al. 2002).

The TRIM-defined compartments contain many other proteins that either interact or simple co-localize with the TRIM proteins. It has been observed that, in addition to homo-interaction, some TRIM proteins hetero-interact or co-localize with each other, e.g. TRIM1-TRIM18, TRIM6-TRIM8, TRIM1-TRIM3. It is also noteworthy that many of the non-TRIM partners are proteins involved in ubiquitylation or contain domains present in proteins belonging to this pathway, often other RING-containing proteins. This point is important because E3 enzymes can in turn be regulated through ubiquitylation and be substrates of other E3s or of themselves. Some TRIM proteins have been observed to mediate their auto-ubiquitylation *in vitro* and hetero-interacting TRIM proteins might regulate their ubiquitylation. In addition, TRIM proteins may play a role in the “non-proteolytic” function of ubiquitin and ubiquitin-like modification. TRIM19/PML is the principal component and organizer of nuclear bodies (NB) to which a conspicuous

number of proteins localize. PML is associated to various cellular process, apoptosis, senescence and cellular viral response through epistatic transcriptional regulation and interactions with many partners such as p53 and Rb (Jensen, Shiels et al. 2001; Pearson, 2001 #652; Pearson and Pelicci 2001). A component of NB is SUMO-1, one of the first UBL proteins discovered (see paragraph 1.2) and PML is modified by SUMO. A subgroup of TRIM protein, known as Transcription Intermediary Factors 1 (TIF1), has been implicated in epigenetic mechanisms of transcriptional regulation involving histone modifiers and heterochromatin-binding proteins (Le Douarin, Zechel et al. 1995). These TRIM proteins may be involved in regulating transcription through a basic mechanism that implicates ubiquitin-dependent transcriptional processes.

Since mutations in TRIM genes result in several pathological conditions, these events could be linked to their E3 Ubiquitin Ligase activity. Two interesting examples in which E3 activity and physiological substrate have been identified are MID1/TRIM18 and TRIM32. The first is responsible for a rare genetic syndrome, X-linked Opitz Syndrome (OS), a congenital human disease shown to result from mutations in the MID1 gene and that affects midline development (Cainarca, Messali et al. 1999). OS-causing mutations are scattered along the entire length of the MID1 gene and affect the ability of its protein product to bind microtubules (Schweiger, Foerster et al. 1999). Moreover, MID1 B-box domain region binds Alpha4, a regulatory subunit of the PP2A-type phosphatases including the principal cellular phosphatase, protein phosphate 2A (PP2A) (Chen, Peterson et al. 1998). The implication of MID1 in the Alpha-4-mediated regulation of phosphatase activity may provide valuable clues as to the pathophysiological consequences of MID1 mutations that underlie Opitz syndrome (Short, Hopwood et al. 2002). In the case of TRIM32, within its fourth NHL repeat, a mutation of an evolutionarily conserved aspartic acid to asparagines is linked to the development of Limb-Girdle Muscular Dystrophy type 2H (LGMD2H),

an autosomal recessive myopathy (Frosk, Weiler et al. 2002). TRIM32 is primarily expressed in skeletal muscle and its level is significantly elevated in muscle undergoing remodelling due to changes in weight bearing. Furthermore, expression of TRIM32 is induced in myogenic differentiation and it associates with skeletal muscle thick filaments, interacting directly with the head and neck region of myosin (Kudryashova, Kudryashov et al. 2005). TRIM32 ubiquitylates actin, acting as an E3 Ubiquitin Ligase, and associates with myofibrils suggesting its likely participation in myofibrillar protein turnover, especially during muscle adaptation (Kudryashova, Kudryashov et al. 2005).

As expected for their role in the ubiquitin pathway, TRIM proteins are involved in several physiological and pathological conditions. Studies on the down-stream target proteins will provide further insight into the molecular mechanism underlying human disease associated to this family.

CHAPTER 2: AIM OF THE PROJECT

The TRIM family represents a sub-class of RING finger proteins that are characterized by the presence of the TRIpartite Motif, which consists of a RING domain, one or two B-box motifs and a coiled-coil region (Reymond, Meroni et al. 2001). In humans, the TRIM family has 68 members and they are involved in many cellular processes such as apoptosis, cell cycle regulation, and innate cellular response to retroviral infections. The presence of the RING domain and its strong association to ubiquitylation suggest a role for this protein family in the ubiquitylation process as E3 ubiquitin ligases.

The E3 enzyme catalyzes the most important step in the ubiquitylation cascade: the interaction with the ubiquitin charged E2 and the substrate. TRIM proteins represent the largest subfamily of the RING domain putative E3 ligases. Indeed, previous studies have demonstrated the E3 function for some TRIM family members, e.g. TRIM23/ARD1, TRIM11, TRIM18/Mid1, TRIM21/Ro52, TRIM25/Efp, and TRIM32 (Meroni and Diez-Roux 2005).

During the ubiquitylation process, target protein specificity is provided by the E3, whereas the E2 and E3 combination determines the topology and length of the ubiquitin chains that will determine the fate of the substrate (degradation of misfolded protein, cell signalling, transcription, regulation of cell cycle, etc). However, to date very little is known about the specificity of human E2/E3 RING interactions and how this is achieved and regulated. In the case of the TRIM proteins, even when the E3 activity is assessed, little is known about the specific UBE2 partner usage and if TRIM-UBE2 binding correlates with different TRIM RING sequence.

The aim of my thesis is to provide a well-defined analysis of the interactions between TRIM family members and E2 enzymes assessing their E3 activity. These data could give an important indication to understand how E2-TRIM complexes might build different types of ubiquitin chains that will determine substrate fate.

Moreover, the involvement of some TRIM family members in specific UBLs (*i.e.* TRIM25-ISGylation and TRIM19-SUMOylation) raises the question of whether all TRIM family members might act as UBL E3 ligases. In particular I will investigate TRIM proteins specific involvement in SUMOylation pathway whose known targets are nuclear proteins with important roles in regulating transcription, chromatin structure and DNA repair.

These results could strongly indicate a role of the TRIM proteins as E3 Ubiquitin/Ubiquitin Like Ligase suggesting a possible usage of TRIM family as model to study the cross-talk between ubiquitylation and SUMOylation.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cloning strategy

The full-length cDNAs of twenty-six Ubiquitin Conjugating Enzymes E2 (UBE2) (TABLE 2) were cloned into pEG202 and pJG4-5 vectors (see paragraph 3.2), which are designed for DNA expression in yeast, and into pCDNA3-HA and pCDNA3-MycGFP vectors (see paragraph 3.3), which are designed for protein expression in mammalian cells; the full-length cDNAs of six TRIM proteins were cloned into pMal c2x vector (see paragraph 3.4), which is designed for protein expression in *E. coli* cells.

3.1.1 RNA extraction

Total RNA for each time point was extracted from Hela and HEK 293 cells with the RNeasy mini Kit (Qiagen). Total RNA was quantified by spectrophotometer analysis, diluted to 100ng/μl final concentration, and checked by UV detection after running 4μl of each sample with 1μl of 5x RNA loading buffer (10ml solution: 80μl of 500mM EDTA pH 8, 720μl of 37% formaldehyde, 2ml glycerol, 3084μl formamide, 20mM 3-[N-morpholino] propanesulfonic acid (MOPS), 5mM sodium acetate, 1mM EDTA, and some bromophenol blue powder, in 10ml final volume of RNase-free water) on a formaldehyde gel (1.2g agarose, 20mM 3-[N-morpholino]propanesulfonic acid (MOPS), 5mM sodium acetate, and 1mM EDTA in 100ml final volume of RNase-free water, and brought at pH 7.0 with NaOH) run in a formaldehyde gel running buffer (20ml of 37% formaldehyde, 20mM 3-[N-morpholino]propanesulfonic acid (MOPS), 5mM sodium acetate).

cDNA was prepared using 1µg of total RNA, 200pmol random hexamers (Invitrogen) and Omniscript reverse transcriptase (Qiagen) in 30µl final volume according to manufacturer's instructions

3.1.2 Production of insert DNA fragments

To clone all genes, Polymerase Chain Reaction (PCR) was performed using primers with restriction site-containing tails; usually, the forward primer carries either EcoRI or BamHI sites upstream of the ATG start codon, which keeps the cDNA in frame with the different tag sequences, and an XhoI site downstream of the stop codon. PCR was performed in a volume of 50µl samples, using 100ng of cDNA obtained from RNA as described above, 500nM forward and reverse primers, 5µl of 10X Vent DNA Polymerase buffer, 0.2mM dNTPs and 0.5µl (1U) of the high fidelity Vent DNA Polymerase (New England Biolabs). The amplification reaction was carried out as follows:

- 1min at 95°C
- 1 min at 95°C
- 30 sec at 56°C
- 4 min at 75°C
- Steps 2 to 4 are repeated 30 times
- 1 cycle: 10 min at 75°C

In order to check the PCR reaction, 5µl of each sample were loaded on a 1% agarose gel. Positive samples were subjected to protein extraction by adding 1 volume (Vol) of phenol/chlorophorm, and centrifuging 1 min at 14,000 in a bench centrifuge at room temperature (RT). The upper phase was collected and DNA was precipitated in 2.5Vol 100% ethanol and 1/10 Vol of

3M Sodium Acetate pH 4.8. After centrifuging 5min at 14,000 rpm at RT, the pellet was washed with 100µl of 70% ethanol and let dry at RT.

3.1.3 DNA restriction

In order to restrict the fragment tails at the EcoRI and XhoI sites or BamHI and XhoI sites, the amplified DNA was incubated as follows: 10-15µl of DNA, 3µl of 10X EcoRI or BamHI buffer, which are both compatible also for the XhoI restriction enzyme, 1µl of EcoRI or BamHI and 1µl of XhoI restriction enzymes (New England Biolabs), and distilled water to a final volume of 30µl. The samples were incubated at 37°C for 2 hrs. The samples were loaded on a 1% agarose gel and the DNA bands corresponding to the expected size were cut from the gel with a razor blade and extracted using the Qiaex extraction kit (Qiagen), according to the manufacturer's protocol, and resuspended in 25µl of 1X TE buffer (10mM Tris-Cl, pH7.5, 1mM EDTA). Extraction efficiency was determined by loading 5µl on an agarose gel. Parallel to this, the pCDNA3-HA, pCDNA3-MycGFP, pEG202, pJG4-5 and pMal c2x vectors were restricted with either EcoRI-XhoI or BamHI-XhoI restriction enzymes, purified by gel extraction as reported above and quantified on a gel.

3.1.4 DNA ligation

After diluting insert and vector DNA at the final concentration of approximately 100ng/μl, the ligation reactions were carried out in final 20μl sample volume, adding 2μl 10X ligation buffer, 1μl vector DNA, 3-10μl insert DNA and 0.5μl T4 DNA Ligase (New England Biolabs), and incubated over night at 16°C. 10μl of each reaction was used to transform competent DH5α bacterial cells.

3.1.5 DNA transformation

In order to introduce the constructs into the bacterial host cells, 50μl of chemical competent DH5α *E. coli* cells, with an efficiency of at least 10⁶ colonies/μg DNA, were incubated for 20 min on ice with 10μl of ligation samples, followed by 2 min thermal shock at 42°C; the cells were incubated for 5 min on ice and then 1 ml LB was added and cells were incubated 1 hr at 37°C in a water bath, centrifuged 5 min at 4,500rpm, resuspended in 20μl LB and plated on 100mm diameter B-agar Petri dishes containing 100μg/mg ampicillin, which allows the selection of the transformed colonies, as the used vectors confer resistance to this antibiotic.

3.1.6 Purification of plasmid DNA

Plates were incubated over night (o.n.) at 37°C and the colonies were picked and grown o.n. at 37°C in liquid LB medium and ampicillin. Plasmid DNA preparations were performed using the Qiagen Mini preparation kits, according to the manufacturer's instructions, which provided small amounts of purified DNA. After plasmid DNA purification, the constructs were analyzed by restriction with either EcoRI-XhoI or BamHI-XhoI enzymes. DNA from positive clones was prepared on a larger scale using Qiagen Midi preparation kits, according to the manufacturer's instructions. Correct constructs were analyzed by sequencing (service provided by PRIMM).

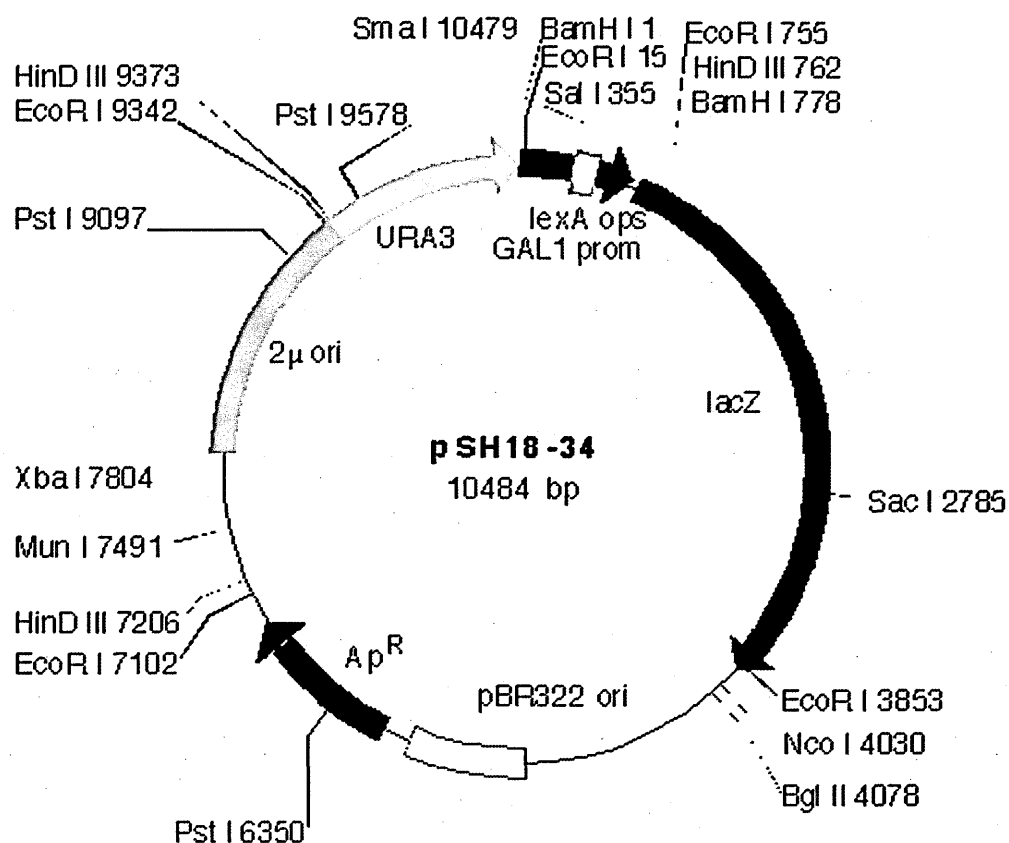
3.2 Interaction Mating

The Interaction Mating is a two-hybrid system used to detect interactions between known proteins. In the Interaction Mating assay two proteins are expressed in yeast: one (the “bait”) contains a DNA-binding moiety (see pEG202 vector); the other (“activation tagged” or “prey”) contains a transcription activation domain (see pJG 4-5 vector). If the two proteins interact, the complex activates transcription of a reporter gene that contains a binding site for the DNA-binding domain of the bait. The Interaction Mating uses *Escherichia coli* LexA repressor as the DNA-binding moiety and two different reporter genes: *LEU2* and *LacZ*, each of which contains upstream LexA operators (see 3.2.1 paragraph).

The Interaction Mating technique relies on the fact that haploid yeast have two different mating type: MATa (EGY42 strain) and MAT α (EGY48 strain), which fuse to form diploids (for details see paragraph 4.1.1). Whereas the yeast EGY48 strain has an integrated *LEU2* reporter gene with its upstream regulatory region replaced by LexA operators (see paragraph 4.1.1), the yeast EGY42 strain contains a LexAop-lacZ reporter that resides on a third plasmid: pSH18-34 (**Figure 9**).

pSH18-34 contains the *URA3* gene and the GAL1, transcription start, and a small part of the GAL1 coding sequence fused to *lacZ*. Reporters for measuring activation are derived from pLR1 Δ 1, in which the GAL1 upstream activation sequences (UASG) have been deleted. Various numbers and types of LexA operators have been inserted in place of UASG to create *lacZ* reporters with different sensitivities. In particular, pSH18-34 contains four of these *colE1* operators and so it is more sensitive than the other ones to activation by LexA fusions and activation-tagged proteins that interact with them (Finley and Brent 1994).

Figure 9



A schematic representation of pSH18-34. pSH18-34 contains a yeast origin of replication (2 μ m ori), the yeast selectable marker gene (URA3), and the GAL1 TATA, transcription start, and a small part of GAL1 coding sequences fused to *LacZ*. It also contains an *E. coli* origin of replication (pBR322ori) and the ampicillin resistance gene (Amp^R).

3.2.1 Cloning the Ubiquitin Conjugating Enzyme cDNAs into vectors for expression in yeast

To perform the Interaction Mating between TRIM proteins and Ubiquitin Conjugating Enzymes (UBE2), I cloned the cDNA of 26 of the best studies E2 enzymes (**TABLE 2**) in the pEG202 and pJG4-5 vectors; 42 TRIM cDNAs had already been cloned in both vectors (Reymond, Meroni et al. 2001). I retrieve the cDNA sequences of known and novel UBE2 enzymes from genomic and expressed sequence databases (at web page www.ncbi.com). Then, as summarized in paragraph 3.1, I designed specific primers with restriction site-containing tails and I performed Polymerase Chain Reaction (PCR) experiments to amplify each UBE2 cDNA. After restricting fragments and plasmids, I performed a ligation reaction to clone the cDNA into the pEG202 plasmid and pJG4-5 plasmid.

pEG202 (**Figure 10**) (Gyuris, Golemis et al. 1993) is a yeast *E.coli* shuttle vector that contains a yeast expression cassette that include the promoter from the yeast *ADHI* gene (*PADH1*), sequences that encode amino acids 1-202 of bacterial repressor protein LexA, which include the DNA binding and dimerization domains; downstream of the LexA coding region unique *EcoRI*, *BamHI*, *Sall*, *NcoI*, *NotI*, and *XhoI* cloning sites are present as well as the transcription terminator sequences from the yeast *ADHI* gene (*TADH1*). It also contains an *E.coli* origin of replication (*pBR ori*), the ampicillin resistance gene (*AmpR*), a yeast selectable marker gene (*HIS3*), and a yeast origin of replication (*2μm ori*). UBE2 cDNAs have been inserted in frame with LexA into the unique restriction sites shown (**Figure 10**) to produce a LexA-UBE2 fusion protein.

pJG4-5 (**Figure 11**) (Gyuris, Golemis et al. 1993) is a yeast *E.coli* shuttle vector that contains a yeast expression cassette that includes the inducible promoter from the yeast *GAL1* gene (*Pgal1*), an ATG followed by 105 codons encoding 9 amino acids from the SV40 large T nuclear

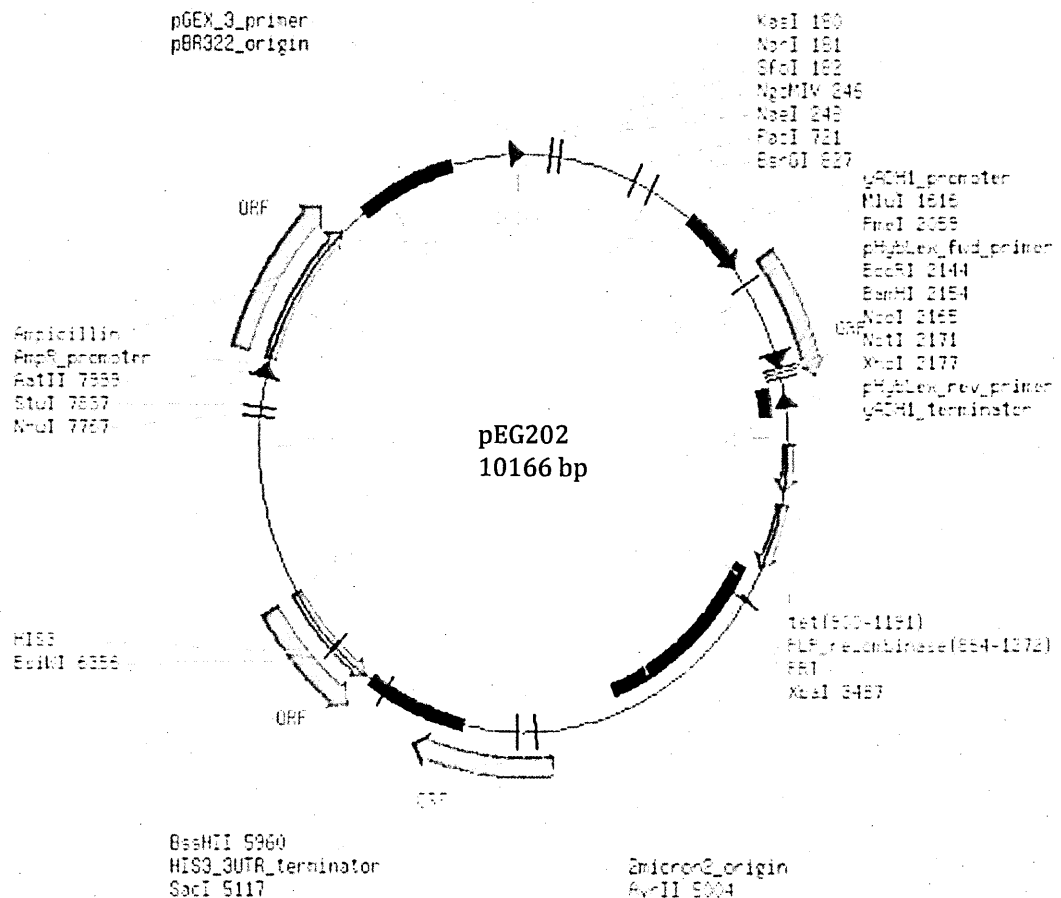
localization signal, 87 amino acids that make up the activation domain B42, and 9 amino acids comprising the haemagglutinin (HA) epitope tag, and the transcription terminator sequences from the yeast ADH1 gene (TADH1). The plasmid also contains an *E.coli* origin of replication (pUC ori), the ampicillin resistance (AmpR), a yeast selectable gene marker (TRP1), and a yeast origin of replication (2 μ m ori) (**Figure 11**. UBE2 cDNAs have been inserted in frame with HA tag into the unique restriction sites shown (**Figure 11**) to produce a HA-B42-UBE2 fusion protein.

TABLE 2

Official Symbol	Other Alias	Gene ID
UBE2A	HHR6A, RAD6A, UBC2	7319
UBE2B	HR6B, UBC2, RAD6B, HHR6B, E2-17KDa	7320
UBE2C	RP3-447F3.1, UBCH10, dJ447F3.2	11065
UBE2D1	E2(17)KB1, SFT, UBC4/5, UBCH5, UBCH5A	7321
UBE2D2	E2(17)KB2, PUBC1, UBC4, UBC4/5, UBCH5B	7322
UBE2D3	E2(17)KB3, MGC43926, MGC5416, UBC4/5, UBCH5C	7323
UBE2D4	HBUCE1	51619
UBE2E1	UBCH6	7324
UBE2E2	FLJ25157, UBCH8	7325
UBE2E3	UBCH9, UbcM2	10477
UBE2F	MGC105540, RGD1307608	363284
UBE2G1	E217K, UBC7, UBE2G	7326
UBE2G2	1110003O05Rik, D10Xrf369, UBC7, Ubc7p	22213
UBE2H	E2-20K, UBC8, UBCH, UBCH2	7328
UBE2J1	CGI-76, HSPC153, HSPC205, NCUBE1, Ubc6p	51465
UBE2K	AW492011, D5Erttd601e, E2-25k, Hip2, Hypg, Lig	53323
UBE2L3	E2-F1, L-UBC, UBCH7, UbcM4	7332
UBE2L6	MGC40331, RIG-B, UBCH8	9246
UBE2M	UBC-RS2, UBC12, hUbc12	9040
UBE2N	UBC13	541130
UBE2Q1	GTAP, NICE-5, PRO3094, UBE2Q	55585
UBE2R1	cdc34	216150
UBE2S	E2-EPF, E2EPF, EPF5	27338
UBE2T	HSPC150, PIG50	29089
UBE2V1	CROC1; AI256840; 0610011J09Rik; Ube2v1	66589
UBE2V2	MMS2, UEV-2, UEV2	7336

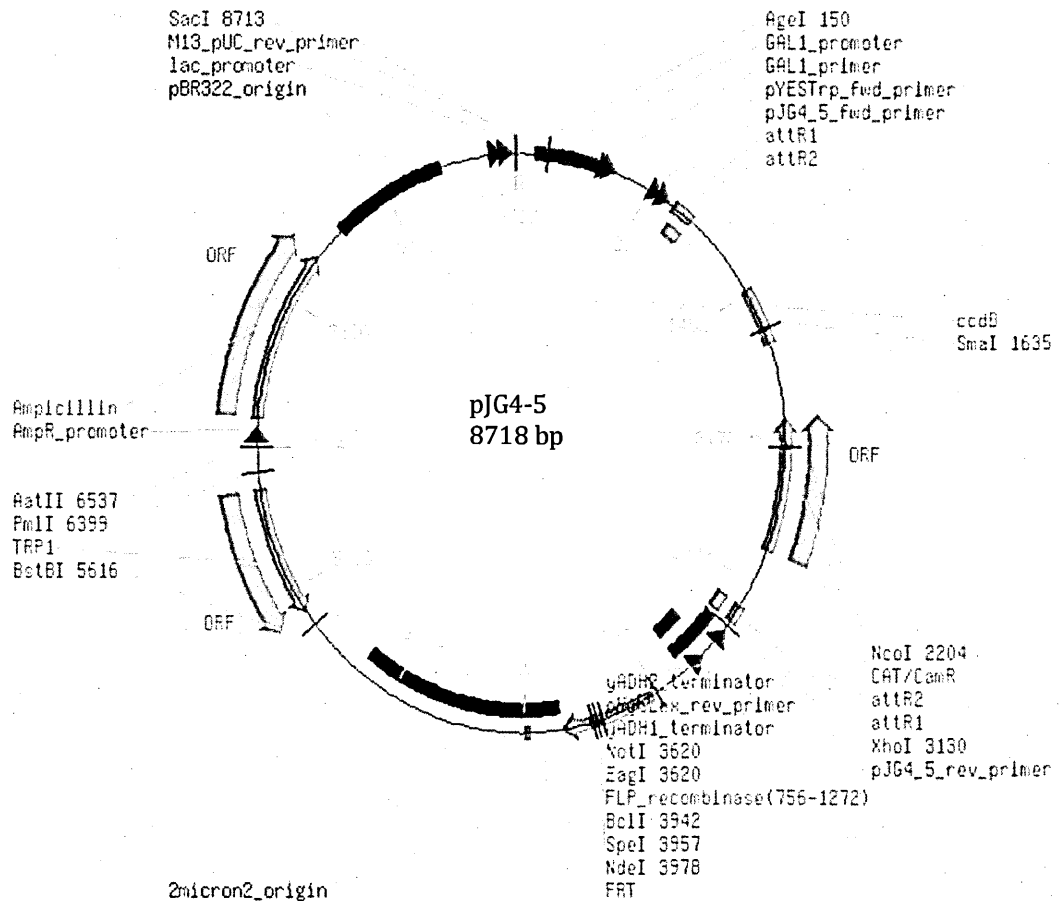
List of Ubiquitin Conjugating Enzymes used in the Interaction Mating assays.

Figure 10



The pEG202 vector. pEG202 is a multicopy yeast plasmid containing the yeast expression cassette with the promoter of the yeast ADH1 (ADH1 promoter), followed by sequences that encode amino acids 1-202 of bacterial repressor protein LexA. It also contains ADH1 terminator, a yeast origin of replication (2 μ m ori), a yeast selectable marker (HIS3), the ampicillin resistance gene (AmpR) and an *E. coli* origin of replication (pBR ori)

Figure 11



The pJG4-5 vector. pJG4-5 is a yeast *E.coli* shuttle vector that contains a yeast expression cassette with the promoter of the yeast *GAL1* gene (P *GAL*), followed by sequences that encode the 106 amino acid fusion moiety that includes the nuclear localization signal from SV40 virus large T antigen, the B42 transcription activation domain, and the haemagglutinin (HA) epitope tag. This plasmid also includes an *E.coli* origin of replication (pUC ori), the ampicillin resistance (AmpR), a yeast selectable marker gene (TRP1), a yeast origin of replication (2 μ m ori) and the transcription terminator sequences from the yeast ADH1 gene (T ADH1).

3.2.2 DNA transformation in yeast

The yeast culture was grown in 100 ml of YPD medium (10g yeast extract, 20g peptone, 900ml of deionised H₂O) at 30°C, with shaking (150 r.p.m.) to an OD₆₀₀ of 1.0, corresponding to about 3 x 10⁷ cells/ml. Yeast cultures were centrifuged at 3500 r.p.m. for 5 minute and the supernatant was poured off. Cells were washed in 10 ml of sterile water and centrifuged again at 3500 r.p.m. for 5 minute. Then, the yeast pellet was resuspended in 5 ml of sterile LiOAc buffer (0.1M LiOAc, 10mM Tris-HCl pH 8.0, 1mM EDTA); cells were centrifuged and the supernatant poured off. The pellet was resuspended in 2 ml of sterile LiOAc buffer; DMSO was added to 10% final concentration. 50µl of this suspension was aliquoted into sterile microcentrifuge tubes containing 1µl of plasmid DNA and 2.5µl of carrier DNA (single-stranded salmon sperm DNA). After adding 300µl of 40% PEG4000 in LiOAc buffer, the tubes were incubated at 30°C for 30 minute. Cells were heat shocked at 42°C for 15 minute and then were plated on 100mm Petri dishes.

PJG4-5, encoding TRP1, was introduced into yeast strain EGY48 (MAT α , TRP1⁻, *his3*⁻ and Leu⁻) and transformants were selected on glucose plates lacking tryptophan (gluc -trp plates) (6.7 g yeast nitrogen base without tryptophan, 20g agar, 850 ml sterile H₂O, 10ml sterile glucose 20%). Instead, pEG202, encoding *HIS3*, was introduced in yeast strain EGY42 (MAT α , HIS3⁻, *trp1*⁻ and Leu⁻) and along with a URA3 *lacZ* reporter, pSH18-34, and transformants were selected on glucose plates lacking uracile and histidine (gluc -ura -his plates) (6.7 g yeast nitrogen base without uracile and histidine, 20g agar, 850 ml sterile H₂O, 10ml sterile glucose 20%).

3.2.3 Yeast Cell lysates

For every TRIM and UBE2 protein transformed in both EGY42 and EGY48 yeast strain, a single colony was picked from the specific plate and resuspended in 10 ml of selective dropout media overnight (o.n.) in a microcentrifuge tube. For EGY42 transformants glucose medium lacking uracile and histidine was used (6.7 g yeast nitrogen base without uracile and histidine, 850 ml sterile H₂O, 10ml sterile glucose 20%); instead, for EGY48 transformants galactose medium lacking tryptophan was used (6.7 g yeast nitrogen base without tryptophan, 850 ml sterile H₂O, 10ml sterile galactose 20%).

Yeast culture was grown to 5 O.D.₆₀₀, spinned for 2 minute, and supernatant was decanted. 200µl of SUTEB buffer (1% SDS, 8M Urea, 10mM Tris pH 8, 10mM EDTA, 0.01% bromophenol blue) was added together with protease inhibitor (SIGMA; 5µl/ml); then, 100µl of 0.5mm Acid Washed Glass Beads (SIGMA) was added and they were vortexed 3 x 45 sec in microcentrifuge. The samples were incubated for 10min at 65°C and the lysate was removed from the beads to a new 1.5ml eppendorf tube. They were centrifuged for 5 min at 12,000 rpm and supernatant was transferred into a new 1.5ml eppendorf tube. A Western Blot (WB) analysis (see paragraph 3.3.3) was performed using anti-LexA (Roche) and anti-HA (Roche) antibodies.

3.2.4 Interaction mating assay

Individual EGY48 transformants were streaked on to standard 100mm Glu -trp plate in parallel lines, six or seven in a plate (**Figure 12**) by applicator sticks. Likewise, individual EGY42 transformants were streaked on Glu -ura -his plates in parallel lines (**Figure 12**). Plates were incubated at 30°C over night (o.n.). The day after, the EGY48 derivatives and the EGY42

derivatives were pressed onto the same replica velvet, so that the streaks from the two plates were perpendicular to each other, and each imprint is lifted with a YPD plate.

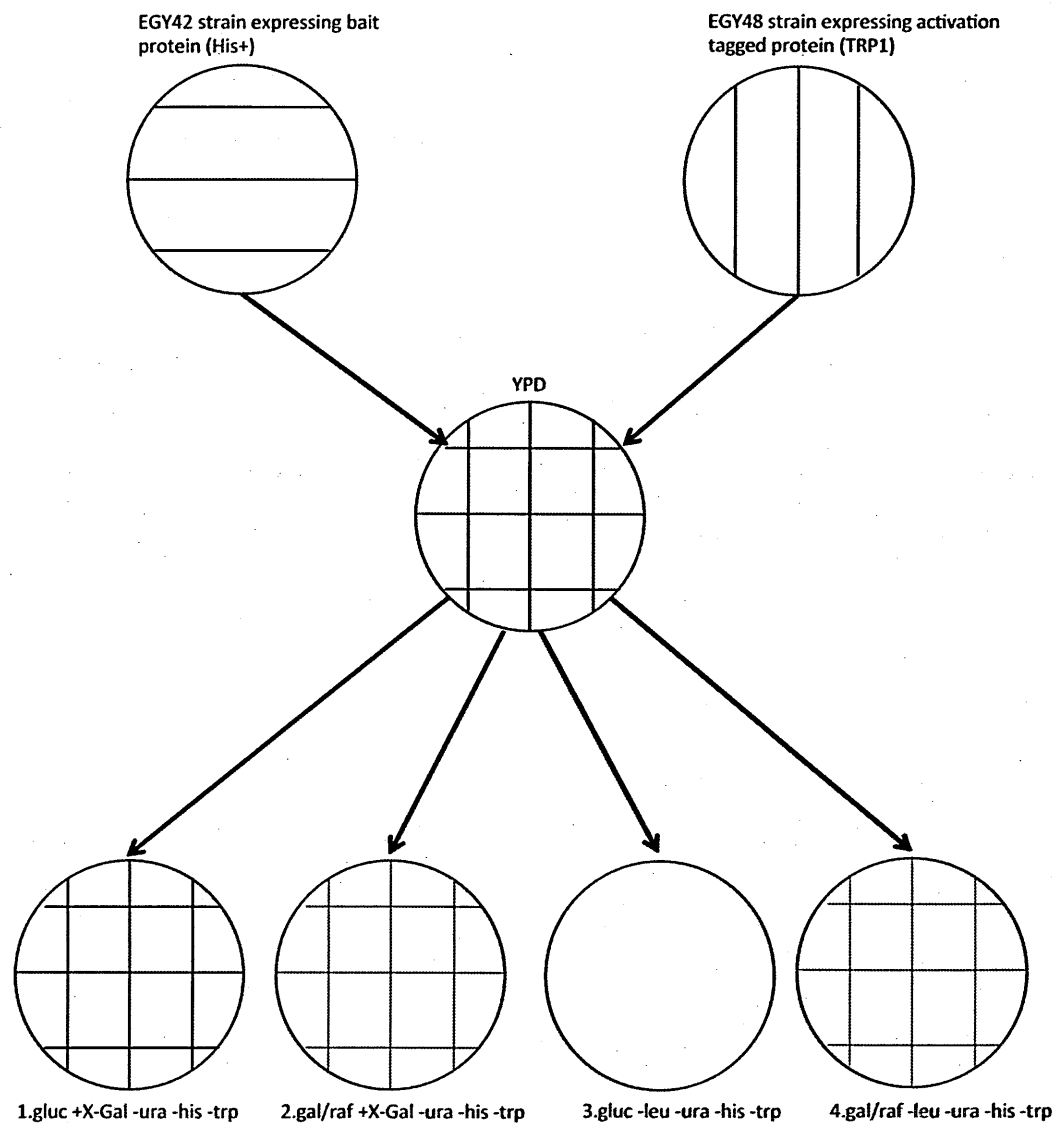
The YPD plates were incubated for 12-20 hr at 30°C, during which time diploids form at the intersections of the two plates.

Replica was done from the YPD plate to the following plates:

1. Glucose plates containing X-gal substrate, but lacking uracile, histidine and tryptophan (gluc +X-Gal -ura -his -trp) (6.7 g yeast nitrogen base without uracile, histidine and tryptophan, 20g agar, 850 ml sterile H₂O, 10ml sterile glucose 20%, 20 µl X-Gal 50mg/ml).
2. Galactose and raffinose plates containing X-gal substrate, but lacking uracile, histidine and tryptophan (gal/raf +X-Gal -ura -his -trp) (6.7 g yeast nitrogen base without uracile, histidine and tryptophan, 20g agar, 850 ml sterile H₂O, 10ml sterile glucose 20%, 10ml sterile raffinose 10%, 20 µl X-Gal 50mg/ml).
3. Glucose plates, lacking leucine, uracile, histidine and tryptophan (glu -leu -ura -his -trp) (6.7 g yeast nitrogen base without uracile, histidine, tryptophan and leucine, 20g agar, 850 ml sterile H₂O, 10ml sterile glucose 20%).
4. Galactose and raffinose plates, lacking leucine, uracile, histidine and tryptophan (gal/raf -leu -ura -his -trp) (6.7 g yeast nitrogen base without uracile, histidine, tryptophan and leucine, 20g agar, 850 ml sterile H₂O, 10ml sterile glucose 20%, 1ml sterile raffinose 10%).

They were incubated at 30°C and examined them after one, two, and three days. Interactors turned blue on X-Gal plates, and grew on gal/raf plates lacking leucine (**Figure 12**).

Figure 12



The Interaction Mating technique. Legends are as follow: 1. Glucose plates containing X-gal substrate, but lacking uracile, histidine and tryptophan (gluc +X-Gal -ura -his -trp); 2. Galactose and raffinose plates containing X-gal substrate, but lacking uracile, histidine and tryptophan (gal/raf +X-Gal -ura -his -trp); 3. Glucose plates, lacking leucine, uracile, histidine and tryptophan (glu -leu -ura -his -trp); 4. Galactose and raffinose plates, lacking leucine, uracile, histidine and tryptophan (gal/raf -leu -ura -his -trp) (for details see the text).

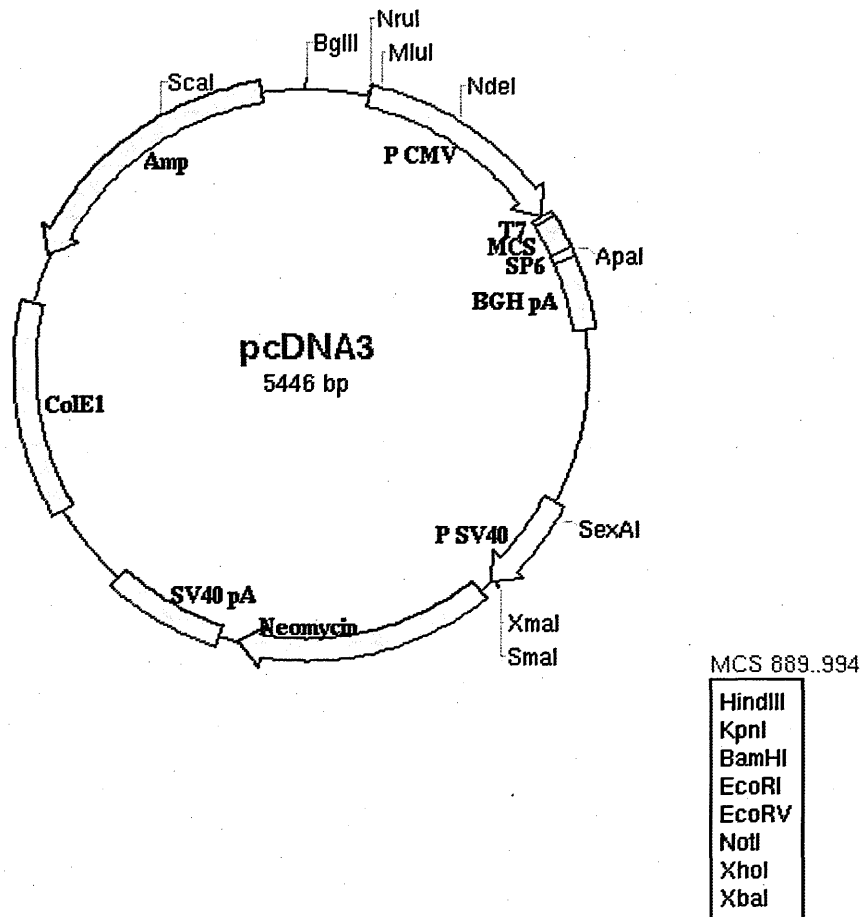
3.3 Cell culture and immunoblot

3.3.1 Cloning the UBE2 cDNAs in vectors suitable for transfection in HEK293 and HeLa cells.

To transfect the UBE2 enzymes in HEK293 and HeLa cells, I cloned the cDNA of the UBE2 enzymes, which are have already been shown in the **TABLE 2**, both in the pcDNA3HA vector and in the pcDNA3myc-GFP vectors suitable for expression in mammalian cells ad easy detectable through the use of the tags. TRIM proteins were already available in both vectors (Reymond, Meroni et al. 2001).

pcDNA3 is a 5446 bp vector for the expression of the proteins in mammalian cells (**Figure 13**). It contains a mammalian expression cassette that includes the Human Cytomegalovirus (CMV) immediate-early promoter/enhancer, which permits efficient, high-level expression of recombinant proteins (Jalanko, Kallio et al. 1989). It also contains the Neomycin resistance gene that induces a selection of stable transfectants in mammalian cells and the SV40 early promoter and origin, which allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen. Moreover, the pcDNA3 vector contains the SV40 early polyadenylation signal, which is necessary for an efficient transcription termination and polyadenylation of mRNA, a pUC origin that allows a high-copy number replication and growth in *E.coli* and an ampicillin resistance gene (β -lactamase) which is important for the selection of vector in *E.coli*. In the pcDNA3-HA and pcDNA3myc-GFP the HA and myc-GFP tag respectively are present upstream the multiple cloning site that allows to clone the UBE2 cDNA in frame with the tags.

Figure 13



The pcDNA3 vector. pcDNA3 is a vector for expression of proteins in mammalian cells. It has a mammalian expression cassette that includes the Human Cytomegalovirus (CMV) promoter/enhancer, a Neomycin resistance gene, the SV40 early promoter and origin. pcDNA3 vector also contains the SV40 early polyadenylation signal, a pUC origin that allows growth in *E. coli* and an Ampicillin resistance gene.

3.3.2 Cell Culture and transient transfection

HeLa and HEK293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) Invitrogen, 100U/ml penicillin/streptomycin and 2mM Glutamine, and split 1:3 every three-four days. Cells were transfected using the Ca^{2+} phosphate transfection method. Typically, 10 μg plasmid DNA was used and 60 μl CaCl_2 2M, 500 μl 2 X HBS solutions, and 10 μl PO_4 . After mixing solution A (cDNA, Ca^{2+} , H_2O to 13 μl final volume) to solution B (2 X HBS, PO_4), CaCl_2 was added (15-20 min at Room Temperature). Then, DNA/ Ca^{2+} phosphate suspension solution was dropped to each 100mm Petri dish containing 1,000,000 cells in a total volume of 9 ml, and they were incubated over night (o.n.) in a 5 % CO_2 incubator.

3.3.3 Immunoblot analysis

Polypeptides separated by SDS/PAGE were transferred to a Hybond-P polyvinylidene difluoride (PVDF) transfer membrane optimised for protein transfer (Amersham Pharmacia Biotech), using a wet blotter (Biorad). After blocking with 5% skim milk in 20 mM Tris pH 7.4 and 150 mM NaCl containing 1% Tween 20, the membranes were probed with the primary antibodies at the dilutions indicated: mouse anti-HA antibody (1:1000; Roche), rabbit anti-LexA (1:1000, Sigma) mouse anti-Myc antibody (1:1000, Santa Cruz), sheep anti-MBP (1:1000, Santa Cruz), or rabbit anti-ubiquitin (1:5000; Bethyl Laboratory). Blots were washed three times with Tris-buffered saline/ Tween 20 0,1% and incubated with either peroxidase-conjugated anti-mouse IgG antibody (1:3000, GE Healthcare), anti-rabbit IgG

antibody (1:3000, GE Healthcare) or anti-sheep IgG (1:3000, GE Healthcare), then developed with a chemiluminescence detection system (ECL, BioFX Laboratories).

3.4 Immunofluorescence

Hela cells were seeded on glass cover slips in 12-multi well plates (Sarsted) the day before transfection with Lipofectamine 2000 (Invitrogen) to obtain low expression level. Twenty-four hours after transfection the cover slips were washed in cold PBS and cells were fixed with 4% paraformaldehyde in PBS for 5 min. Cells were then permeabilized in 0.5% Triton-X-100 in PBS for 10 min. Non-specific binding sites were blocked by incubating with 5% Bovine Serum Albumine (BSA), 0.1% Tween in PBS for 1 hour. Cover slips were then incubated with anti-HA monoclonal antibody (1:500, Roche) for 2 hours. After washing, cover slips were incubated with Cy3-conjugated anti-mouse secondary antibody (1:100, Jackson laboratories). Slides were mounted using Vectashield with DAPI (Vector Laboratories); images were acquired both on LeicaDM2500 and processed with the Leica Application Suite V3 software and on Nikon confocal D-Eclipse C1 imaging microscope with Nikon software and processed either as grey scale or dual colour TIFF images in Adobe Photoshop (Adobe Systems).

3.5 MBP-TRIM protein expression and purification

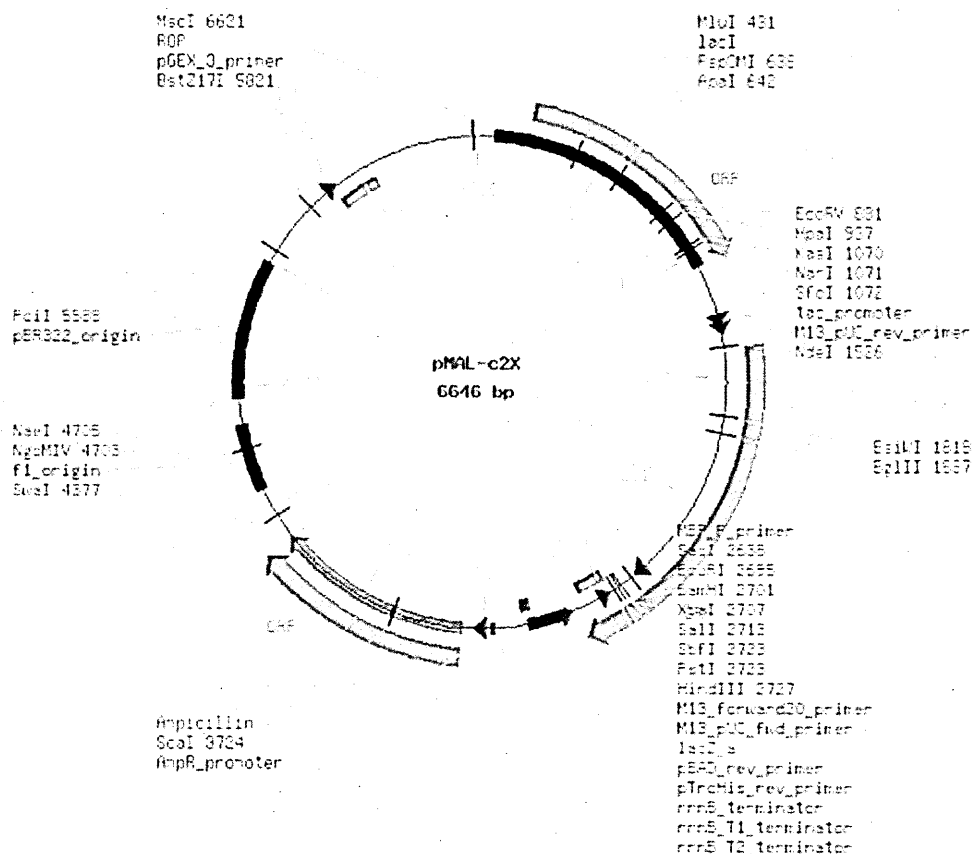
3.5.1 Cloning the TRIM cDNAs in vectors suitable for expression in *E.coli*

TRIM1, TRIM9, TRIM11, TRIM18, TRIM27 and TRIM32 were cloned in pMal c2x vector (NEB Biolabs) as indicated in 3.1 paragraph, which encodes maltose binding-protein (MBP), resulting in the expression of MBP-TRIM fusion proteins in *E.coli* (Duplay, Bedouelle et al. 1984).

pMAL-c2x is a 6646 bp vector for the expression of the proteins in prokaryotic cells (**Figure 14**). The pMAL-c2X vector contains the strong “tac” promoter, the ampicillin resistance that is necessary for the selection of the vector in *E.coli* strain and the *malE* gene, which encodes maltose-binding protein (MBP), fused to the *lacZα* gene. Restriction sites between *malE* and *lacZα* are available for inserting the cDNA selected and insertions usually inactivate the β-galactosidase α-fragment activity of the *malE-lacZα* fusion. This results in a blue to white colour change on Xgal plates when the constructs are transformed into an α-complementing host such as TB1 or JM107 strain. The vector also carries the *lacI^q* gene, which encodes for the *Lac* repressor. This keeps expression from P_{tac} low in the absence of IPTG induction. Moreover, the pMAL-c2X vector also contains the sequence coding for the recognition site of Factor Xa protease, located just 5' to the polylinker insertion sites. This allows MBP to be cleaved from the protein after purification.

All TRIM genes are inserted downstream from the *malE* gene of the *E.coli* resulting in the expression of an MBP-TRIM fusion protein.

Figure 14



The pMAL-c2X vector. pMAL-c2x is a vector for expression of proteins in prokaryotic cells. It has the strong “tac” promoter, the *malE* gene which encodes for the Maltose Binding Protein (MBP) and *lacI^q* gene, which encodes for the *Lac* repressor. It also contains the Ampicillin resistance gene and the sequence coding for the recognition site of Factor Xa protease.

3.5.2 MBP-TRIM protein expression and purification

The DNA of the recombinant MBP-TRIM constructs was transformed into *E.coli Rosetta (DE3)* cells as indicated in 3.1.4 paragraph. Cells from single colonies were grown in LB broth (10 mL) supplemented with ampicillin (100µg/mL) and chloramphenicol (100µg/mL). After an over night (o.n.) of growth at 37°C, the OD₆₀₀ was measured and the cultures were diluted to a final OD₆₀₀ of 0.1 in 1L of fresh LB containing ampicillin and chloramphenicol at the same concentration indicated above. For each TRIM proteins, the cultures were grown for 3.5 hr at 37°C and simultaneously induced with 0.1mM isopropyl β-d-thiogalactoside (IPTG) when the OD₆₀₀ of all cultures was 0.6–0.8. Before adding IPTG, a 100µl aliquot was removed for Western Blot analysis. The pellet was resuspended in 50µl of sample buffer (250 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 0.002% Bromphenol Blue). Cell cultures were grown at 24°C o.n. Cell cultures were then cleared using a JA-12 Beckman rotor (5,000 rpm, 20 min, 4°C) and cell pellets were resuspended in 40 mL of Lysis Buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.5 mM β-mercaptoethanol and 1mM Benzamidine. Resuspension was achieved by agitating the Beckman centrifuge tubes containing the cells and the buffer on a shaker for 15 min at 600 rpm. The centrifuge tubes were incubated on ice (30 min) and sonicated at 4°C three times (40 sec, 50% amplitude of Misonix 3000 sonicator). Cell lysates were cleared using a JA-12 Beckman rotor (11,000 rpm, 40 min, 4°C) and proteins were purified using an amylose-bound chromatography. The columns were washed three times with Lysis buffer and the proteins were eluted by addition of an elution buffer containing: 50 mM Tris HCl pH7.5, 500 mM NaCl, 10mM Maltose, 5mM β-mercaptoethanol. MBP-TRIM proteins were separated on SDS-PAGE (10% gels) and visualized with Coomassie blue staining.

3.6 MBP pull-down assay

In order to bound MBP-TRIM proteins to amylose beads, 4µg MBP-TRIM protein or 1µg MBP protein was incubated with 20µl of amylose beads (New England Biolabs) in 50mM Tris-HCl pH 7.5 at 4°C for 2 hours. The beads were then harvested (3300 rpm, 3 min, 4°C) and washed three times with Lysis Buffer (20 mM Tris pH7.5, 20% Glycerol, 50mM NaCl, 5mM EDTA pH8, 0.1% Triton, 1.5mM PMSF and 1 mg/ml aprotin, leupeptin and pepstatin). A 10µl aliquot of the mixture was removed for Western Blot analysis and amylose-MBP-TRIM proteins were visualized with Coomassie blue staining. Afterwards, each amylose-MBP-TRIM proteins was incubated with 5µg of HEK293T crude extract transiently transfected with MycGFP-UBE2 enzymes at 4°C for 4 hours in Lysis Buffer. The beads were harvested and washed three times with Lysis buffer; bound proteins were separated on SDS-PAGE and UBE2 were visualized with immunoblotting using the anti-Myc antibody (see paragraph 3.3.3).

3.7 *In vitro* Ubiquitylation assays

The *in vitro* ubiquitylation assays were carried out in a volume of 15 µL containing 34 nM Uba1 (Human Recombinant, Ron T.Hay's lab), 0.8 µM E2-conjugating enzyme (Human Recombinant, Ron T. Hay's lab), 35 nM ubiquitin (Boston Biochem), 5 mM MgCl₂, 2 mM ATP, 150 mM NaCl, 150 mM TCEP, 0.1% NP40 and 2.2 µM MBP-TRIM protein in 50 mM Tris-HCl pH7.5. MBP protein was used at 2 µM. After incubation at 37°C for 2 hrs, the reactions were terminated by the addition of Loading buffer (see above) and analysed by SDS-PAGE (10% gels). Immunoblots using anti-Ubiquitin and anti-MBP antibodies were performed.

3.8 *In vivo Ubiquitylation assays*

HEK293T cells were co-transfected with HA-Ubiquitin (Stefano Gustincich's lab) and MycGFP-TRIM proteins. When applicable, 42 hrs after transfection the culture medium was replaced with fresh medium containing MG132 proteasome inhibitor (Sigma) at a final concentration of 20 μ M and the cells were further cultured for 6 h. The total cell lysate was prepared in RIPA buffer (50 mM TRIS-HCl pH 8, 0.1% SDS, 150 mM NaCl, 0.5% DOC, 1% NP-40, 1.5 mM PMSF and 1 mg/ml aprotinin, leupeptin and pepstatin). DNA in the sample was sheared with a 22-gauge needle and the lysate was centrifuged using a Sigma 12024H rotor (13000, 30 min, 4°C). The supernatant was then incubated with 1 μ g anti-Myc antibody (9E10 Santa-Cruz) over-night at 4°C. After adding protein A-Sepharose beads (Sigma), complexes were pelleted and washed 4 times with lysis buffer. Immunoprecipitates were separated on SDS-PAGE (10% gels) and analyzed by Immunoblotting.

3.9 *In vitro SUMOylation assays*

The *in vitro* SUMOylation reactions were performed at 37°C for 4 hours in a 20 μ l reactions containing 34 nM SAE1/SAE2 (human recombinant, Ron T.Hay's lab), 0.4 μ M Ubc9 (human recombinant, Ron T.Hay's lab), 2.2 μ M MBP-TRIM protein or GST-PML (human recombinant, Ron T.Hay's lab) protein in 50 mM Tris-HCl pH7.5 and 32 μ M SUMO 1 or SUMO2 (Ron T. Hay's lab). The reaction buffer contained 50 mM Tris-HCl pH7.5, 5 mM MgCl₂, 2 mM ATP, 150 mM NaCl, 2.5 mM DTT, 0.1% NP40. The reactions were terminated by the addition of Loading buffer (see above) and analysed by SDS-PAGE (4-12% gradient gels). Immunoblots using anti-SUMO1, anti-SUMO2 and anti-MBP antibodies were performed.

CHAPTER 4: RESULTS

4.1 Preliminary screening of TRIM proteins and Ubiquitin Conjugating Enzymes interactions performed through Interaction Mating.

To act as Ubiquitin Ligase or E3 the interaction with the Ubiquitin Conjugating Enzyme or UBE2s is required. Therefore, I preliminary tested the interaction between TRIM proteins and UBE2 proteins through the Interaction Mating technique, as a first evidence for a putative E3 role of TRIM proteins.

4.1.1 Interaction Mating assay

The yeast Interaction Mating is a form of two-hybrid system to test interaction between known proteins and it offers a number of advantages over biochemical methods such as quickness and sensibility.

The two-hybrid approach takes advantage of the modular domain structure of eukaryotic transcription factors. They usually have at least two distinct functional domains: one that directs binding to specific DNA sequences and one that activates transcription (Finley and Brent 1994). This modular structure is best illustrated by yeast experiments showing that the DNA binding domains or activation domains can be exchanged from one transcription factor to the next and retains function.

The Interaction Mating has three basic components:

- A yeast vectors for expression of a known protein fused to a DNA binding domain (LexA)
- A yeast vector for expression of a known protein fused to a transcription activation domain (B42)
- Yeast reporter genes that contain binding sites for the DNA binding

domain (LexA responsive elements)

The system I used utilizes a yeast strain with two reporter genes. One reporter is a yeast *LEU2* derivative that has its normal upstream regulatory sequences replaced with LexA operators. Transcription of the *LexA-operator-LEU2* gene (*LexAop-LEU2*) is measured by the ability of the strain to grow in the absence of leucine, which requires the *LEU2* gene product. The *LexAop-LEU2* gene is integrated into the yeast chromosome. The other reporter gene is *lacZ*, which provides a secondary assay of activation. When the transcription of *lacZ* gene is activated, the strain is able to form blue colonies on X-Gal medium.

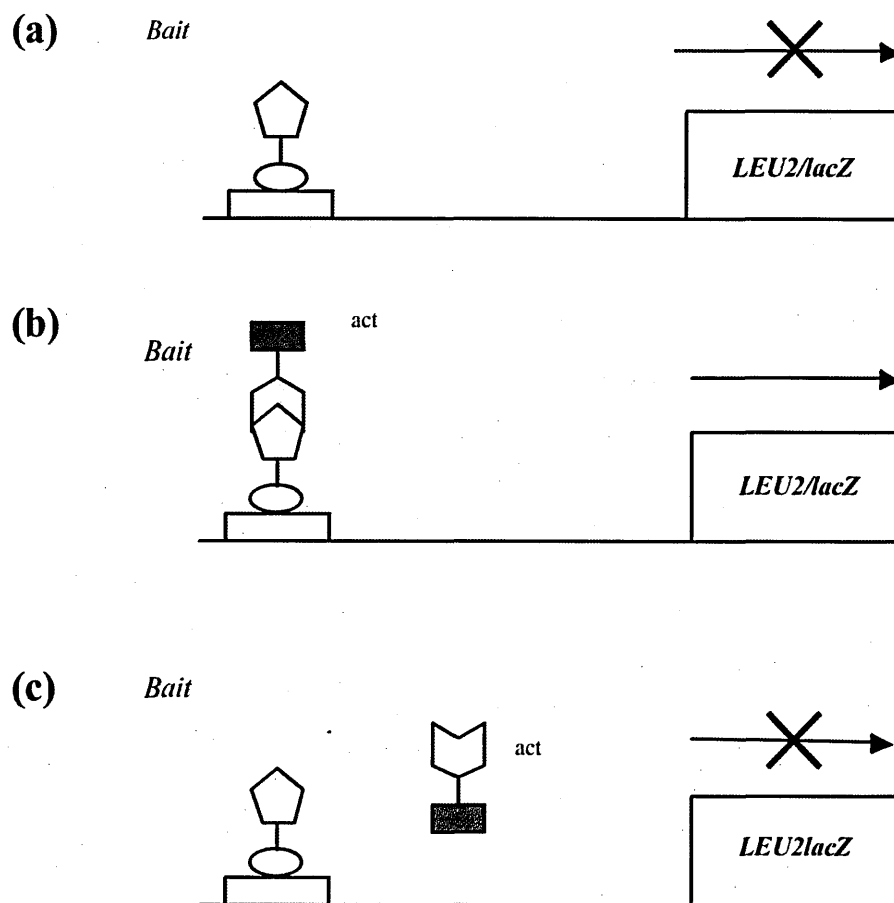
The Interaction Mating technique takes advantage of the fact that haploid cells of the opposite mating type will fuse to form diploids when brought into contact with each other. The activation-tagged protein is expressed in one yeast strain mating type (e.g. MAT α) and the bait is expressed in a second strain of the opposite mating type (e.g. MAT α). When the two strains are mixed on the same plate, they form diploids in which the bait and activation-tagged proteins have the opportunity to interact and activate the reporter genes. The interaction is measured as activation of the *LexAop-LEU2* and *LexAop-lacZ* reporters.

The bait protein is constitutively expressed from the pEG202 plasmid. It binds to LexA operators upstream of the reporter genes, *LEU2* and *lacZ*, but does not activate transcription *per se*.

The activation-tagged cDNA encoded protein is conditionally expressed from the GAL1 promoter. In glucose medium (**Figure 15a**) the activation tagged cDNA encoded protein is not made because the GAL1 promoter is repressed. The yeast does not grow in the absence of leucine and forms white colonies on X-gal plates. Instead, when the yeast is grown in galactose medium, activation tagged cDNA encoded protein is expressed (**Figure 15b** and **Figure 15c**). If the bait interacts with the activation-tagged cDNA encoded protein, the activation domain is tethered to the DNA and

activates the reporter genes. The cells form colonies on a medium lacking leucine and form blue colonies on an X-gal plate (**Figure 15b**). Instead, if the bait does not interact with the activation-tagged cDNA encoded protein, the cells do not grow on a medium lacking leucine and form white colonies on X-gal plate (**Figure 15c**).

Figure 15



Schematic representation of the Interaction Mating. A) Glucose medium. The LexA fusion protein (bait) is made and binds to LexA operators upstream of the two reporter genes, *LEU2* and *lacZ*. The activation-tagged cDNA encoded protein is not expressed because the *GAL1* promoter is repressed in the presence of the glucose. B) Galactose medium: interaction. Galactose induces expression of an activation-tagged cDNA encoded protein that does not interact with bait protein and no activation of the gene is observed. C) Galactose medium: no interaction. Galactose induces expression of an activation-tagged cDNA encoded protein that does not interact with bait protein.

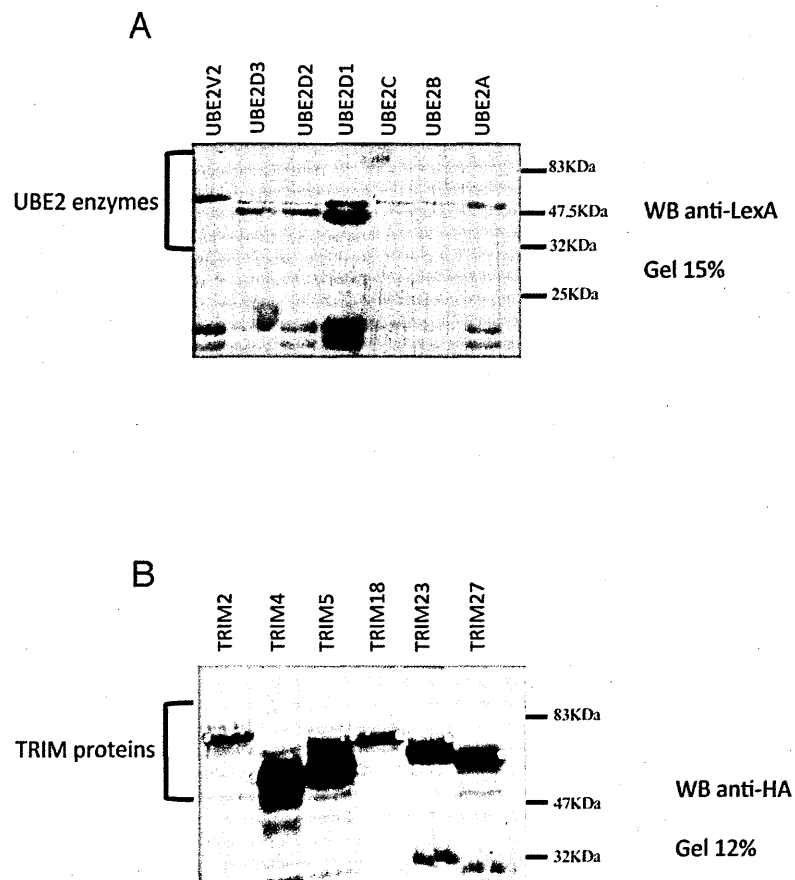
4.1.2 Western Blot analysis of both TRIM and UBE2 protein expression in yeast cell

A Western Blot analysis of TRIM proteins and UBE2 enzymes cloned both in the pEG202 and in the pJG4-5 vectors was performed before all the Interaction Mating experiments to verify the expression of the fusion proteins in the yeast strains.

The monoclonal anti-LexA antibody was used to specifically recognize the LexA tag of the pEG202 vector. The Western Blot assays performed with anti-LexA antibody showed bands of the expected size for both LexA-TRIM (60/100KDa) and LexA-UBE2 (38/48KDa) fusion proteins (**Figure 16a**). Instead, the anti-HA antibody specifically recognized the HA epitope tag present in the pJG4-5 vector. Western Blot analysis performed with anti-HA antibody showed bands of the expected size for both B42-HA TRIM (50-90 KDa) and B42-HA UBE2 fusion proteins (28/32KDa) (**Figure 16b**). Consistently, the Western Blot performed to verify the expression of TRIM proteins in yeast lysates confirmed what already reported by Reymond and collaborators (Reymond, Meroni et al. 2001).

Thus, Western Blot analysis showed that both TRIM and UBE2 fusion proteins were efficiently expressed from both vectors.

Figure 16



Western Blot analysis of TRIM and UBE2 proteins expressed in yeast cells. Here I reported two Western Blot scans that are representative of all Western Blot analysis performed to test both TRIM and UBE2 fusion protein expression in yeast lysates. (a) Western Blot performed using anti-LexA antibody upon a selection of UBE2 enzymes (Molecular Weight: 38-48KDa). (b) Western Blot performed using anti-HA antibody upon a selection of TRIM proteins (Molecular Weight: 56-85 KDa). Red asterisks indicate B42-HA TRIM proteins molecular weight.

4.1.3 The Interaction Mating assay between UBE2 enzymes and TRIM proteins

Thanks to the Interaction Mating technique, I tested 42 TRIM proteins (www.trimbase.tigem.it) expressed both as bait and as prey in the yeast strain EGY48 (mating type α) and in the yeast strain EGY42 (mating type *a*) against almost 26 UBE2 proteins (see **TABLE 2**) expressed in both vectors and yeast strains.

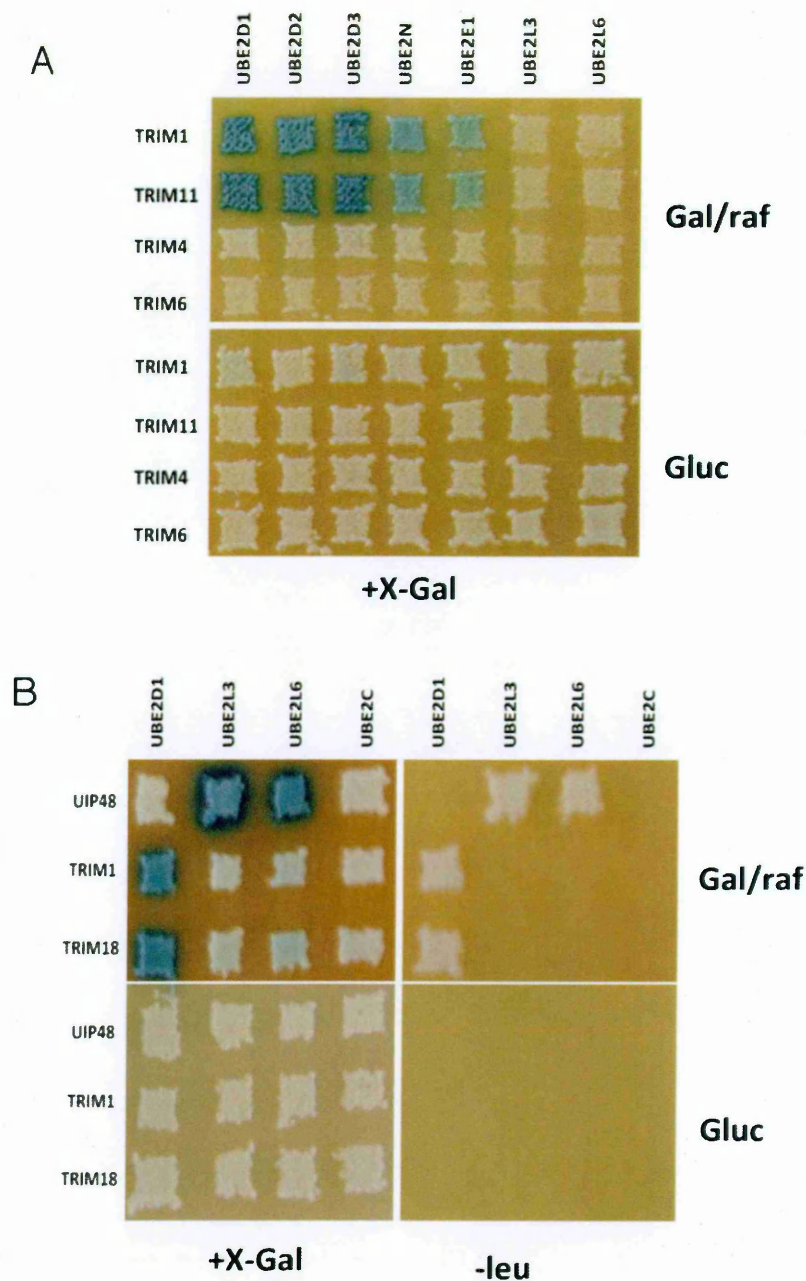
To be certain of the interactions observed between the TRIM proteins and the UBE2 enzymes, I repeated the Interaction Mating assays seven times with freshly transformed TRIM and UBE2 pEG202 and pJG4-5 constructs in yeast strain every time (see paragraph 3.2.1). I summarized the results of the Interaction Mating assays in **TABLE 3**. This analysis revealed more than 100 interactions and demonstrated that the majority of TRIM proteins tested interact with one or more UBE2 enzymes (**TABLE 3**) (see example of Interaction Mating in **Figure 17**). Numerous interactions were observed with the D (D1-4) and E (E1-3) families of UBE2 enzymes while several TRIM proteins also showed binding to UBE2N. Two peculiar interactions were also observed: i) an exclusive binding between TRIM9 and UBE2G2 and ii) the interaction of TRIM32, beside the D and E classes and UBE2N, with UBE2V1 and UBE2V2 (**TABLE 3**). The UBE2 and the TRIM proteins were properly expressed in yeast and lack of the reporter activation mainly underlies real UBE2 selection. Inclusion of UIP48, a RING finger-containing protein able to interact with UBE2L3 and UBE2L6 but not with the D and E families, demonstrated the specificity of the interactions observed (Martinez-Noel, Muller et al. 2001). In some cases however, lack of an interaction may represent intrinsic flaws in the yeast two-hybrid experiment. Indeed, compared to the classical techniques used to evaluate protein-protein interactions (for details see **DISCUSSION** chapter), the main criticism applied to the yeast two-hybrid screen is the high number of false negative.

Many natural protein–protein interactions cannot be detected using the yeast two-hybrid method. Some proteins do not interact in the environment of the yeast nucleus, such as proteins of the secretory compartments that require oxidative conditions or glycosylation for proper folding. Integral membrane proteins are unlikely to work in the context of reconstituted transcription factor and many interactions are triggered by post-translational modifications not available in yeast. In addition, another important limitation in the usage of the yeast two-hybrid approach is the possibility of false positive interactions. The reason for this error rate lies in the principle of the screen: the assay investigates the interaction between overexpressed fusion proteins in the yeast nucleus. It is well demonstrated that overexpression can result in non-specific interactions and some proteins might specifically interact when they are co-expressed in the yeast, although in reality they are never present in the same cell at the same time. As a consequence of these specific issues, independent verification through other alternative techniques (i.e. Co-immunoprecipitations, *in vitro* pull down, Mass spectroscopy, etc.) of a putative protein-protein interaction is essential.

However, the yeast two-hybrid has demonstrated its power by its methodological diversity and technical simplicity to rapidly generate a large amount of reliable protein-protein interaction data. The strength of yeast two-hybrid system is its ability to identify direct interactions and also to detect interactions of lower affinity, which are rather transient.

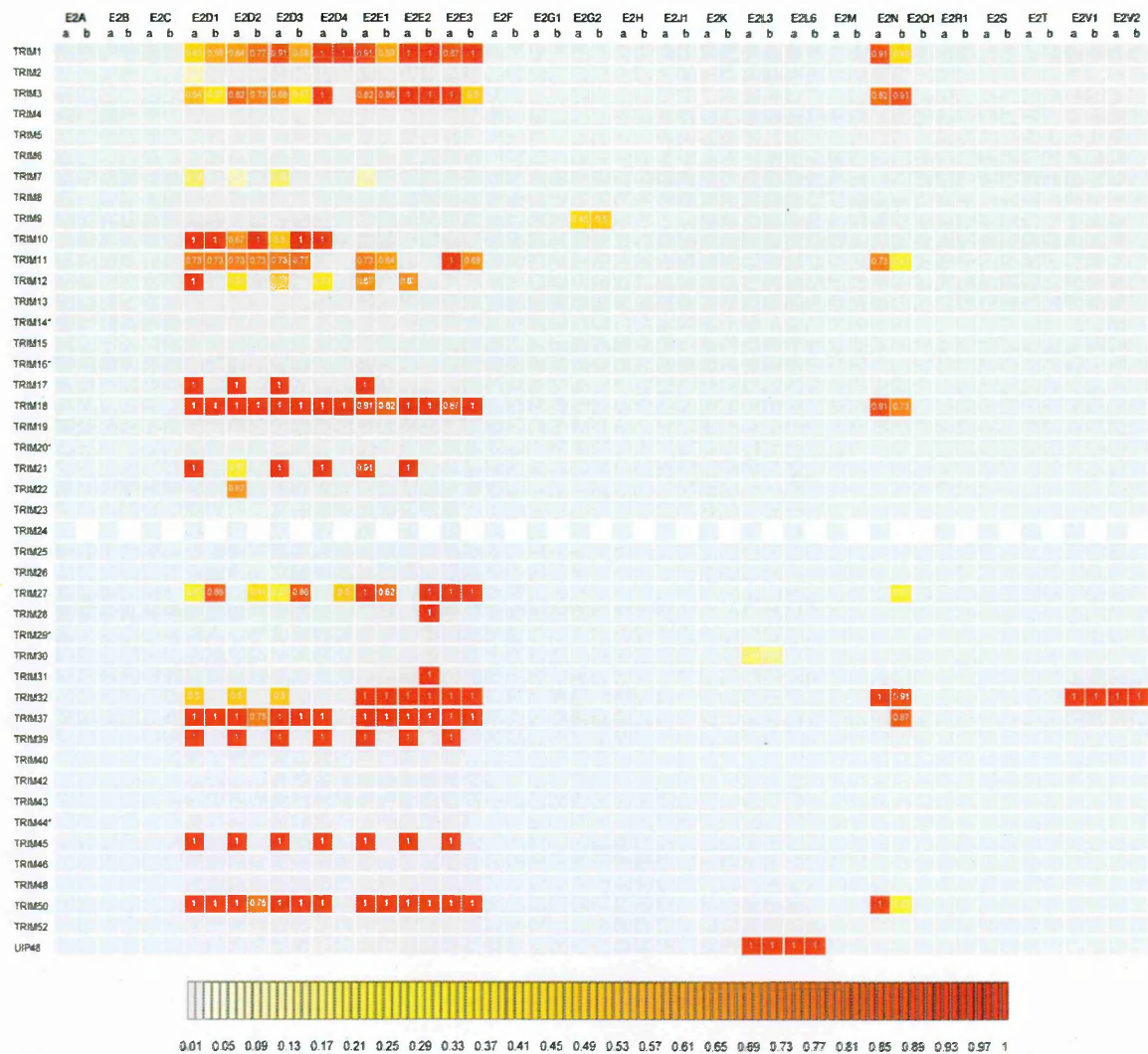
Thus, since the transient nature of E3/E2 interactions, the yeast Interaction Mating approach resulted as the finest technique to investigate putative TRIM/E2 interactions. Even if I confirmed all the TRIM/E2 interactions seven times, as reported in TABLE3, I can not exclude the presence of eventually both false positive and false negative interactions.

Figure 17



Examples of Interaction Mating. A) Interaction Mating assay in which I tested interactions between TRIM proteins and UBE2 enzymes. The interaction is given by colonies turning blue on Galactose/ Raffinose (gal/raf) +X-Gal plates. B) Interaction Mating assay in which I tested interaction between UIP48 and UBE2L3/L6. TRIM proteins are used as control. The interaction is given by colonies turning blue on gal/raf +X-Gal plates and colonies growing on Gal/raf -leu plates (for details see the text). The Interaction Mating screen was repeated seven times. The pictures reported above are representative of the results obtained

TABLE 3



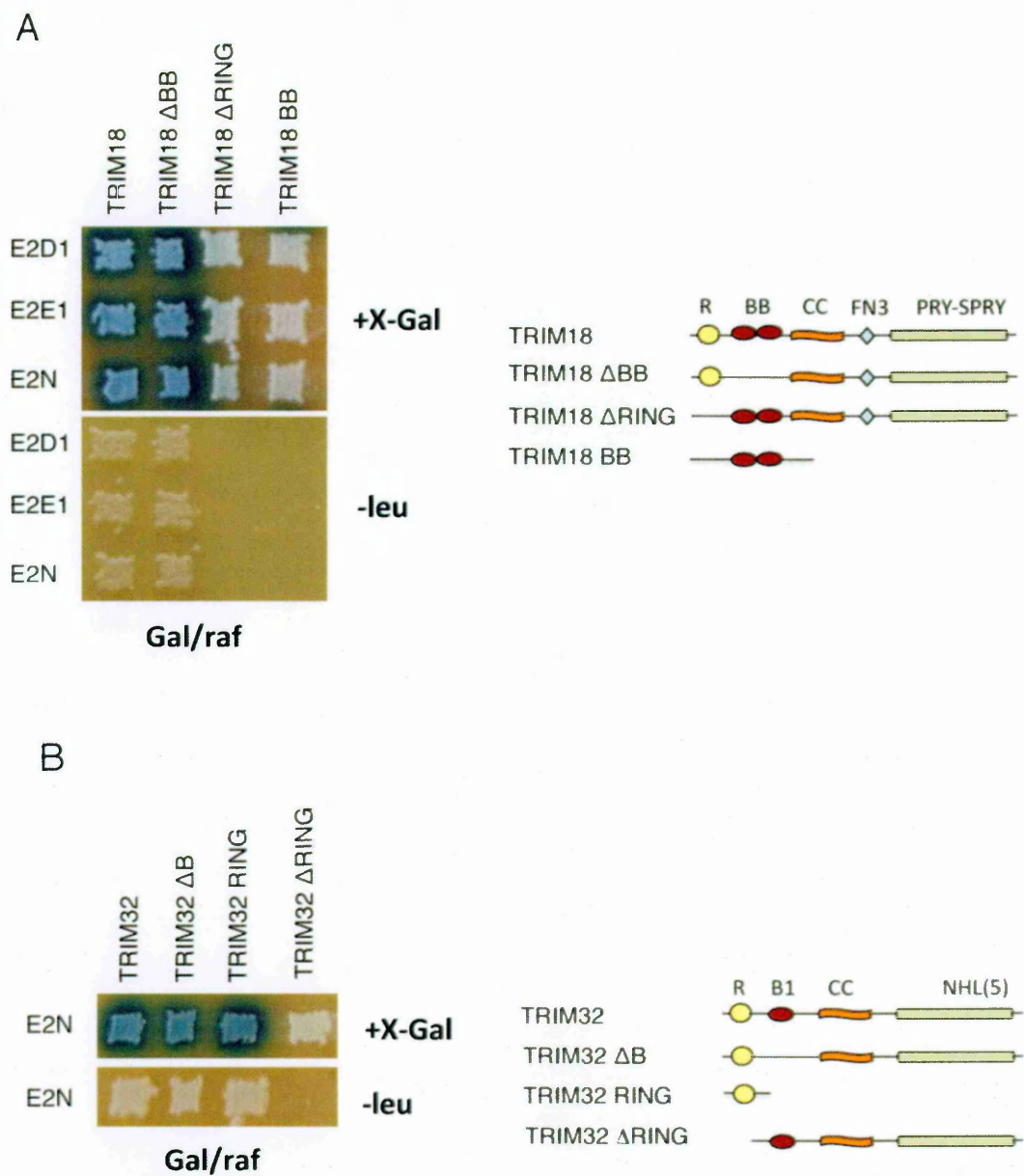
Schematic representation of the results obtained with the yeast Interaction Mating screening. To assure the experiments' reproducibility, I repeated the Interaction Mating experiments seven times. The TRIM and the UBE2 (E2) clones tested are indicated; the letters below the E2s indicate the direction of the two-hybrid experiments: a, B42-BD and b, LexA-DBD. The asterisk indicates the RING-less TRIM proteins. The interaction strength and reproducibility are indicated by arbitrary scored in the range 0-1, represented also by the colour-scale shown at the bottom, which was calculated as the fraction of detected interactions on the number of the experiments (seven performed for each pair tested) multiplied by 0.5 if the interaction was observed with only one reporter gene. Grey cells indicate no interaction; white cells indicated non-tested pairs.

4.1.4 TRIM proteins interact with UBE2 enzymes through their RING domain

It is known that the RING domain underlies ubiquitin ligase activity by directly binding the UBE2 enzymes. This key-role was definitely provided by the crystal structure of Cbl RING domain bound to Ubch7/UBE2L3 (Zheng, Wang et al. 2000) and then by the NMR analyses of the BRCA1 and CNOT4 complexed with Ubch5/UBE2D (Dominguez, Bonvin et al. 2004). Consistently, the Interaction Mating screening revealed the absence of the interaction between the 5 “unorthodox” TRIM members that lack the RING domain (Sardiello, Cairo et al. 2008) and the UBE2 enzymes (**TABLE 3**). Moreover, through the Interaction Mating I also analyzed TRIM18, TRIM32 and TRIM50 mutants carrying individual deletions of relevant protein regions [R, B-boxes (BB), CC, RFP-like and N- or C-terminal regions; **Figure 18**] for the interaction with the UBE2 enzymes. As already reported in literature (Reymond, Meroni et al. 2001) deletion of the CC region resulted in the loss of self-association confirming that the CC region is necessary and sufficient for homo-interaction. Interestingly, deletion of the RING region resulted in loss of the interaction with UBE2 indicating that the RING region is involved in the interaction with UBE2 proteins (**Figure 18a** and **Figure 18b**). As shown in **Figure 18**, deletion of the BB-CC region and RFP region affected neither the homo-interaction nor the interaction with UBE2 proteins. In addition, the RING domain alone is able to interact with UBE2 proteins. Moreover, this analysis also demonstrated that the B-box domains, although structurally similar to the RING domain, were not intimately involved in the basic TRIM/E2 interaction.

Taken together these data indicate that the RING domain is therefore necessary and sufficient for the interaction between TRIM proteins and UBE2 enzymes.

Figure 18



Two-interaction mating panels showing either TRIM18 (A) or TRIM32 (B) domain deleted mutants, shown in the right scheme, against the UBE2 enzymes indicated. Blue colonies in X-gal plates (Xgal) and growth on plates lacking leucine (-leu) in the presence of Galactose/Raffinose (Gal/Raf) represent positive interactions.

4.2 Validation of the interaction between TRIM and UBE2 proteins through MBP pull-down assays.

To validate the TRIM-UBE2 interactions observed through the Interaction Mating screening, I used the MBP pull-down technique. I chose to test TRIM1, TRIM9, TRIM11, TRIM18, TRIM27 and TRIM32 as representative members of the family since they recapitulated the most important interactions observed in yeast Interaction Mating screening.

4.2.1 MBP pull-down assay

MBP pull-down assay is an *in vitro* method used to determine a physical interaction between two or more proteins. The MBP pull-down technique has more advantages such as sensitivity and high accuracy; moreover, either the discovery or the confirmation of protein-protein interactions depends heavily on the nature of the interaction studied. Interactions can be stable or transient, and the characteristic determines the conditions for optimizing binding between the bait and prey proteins.

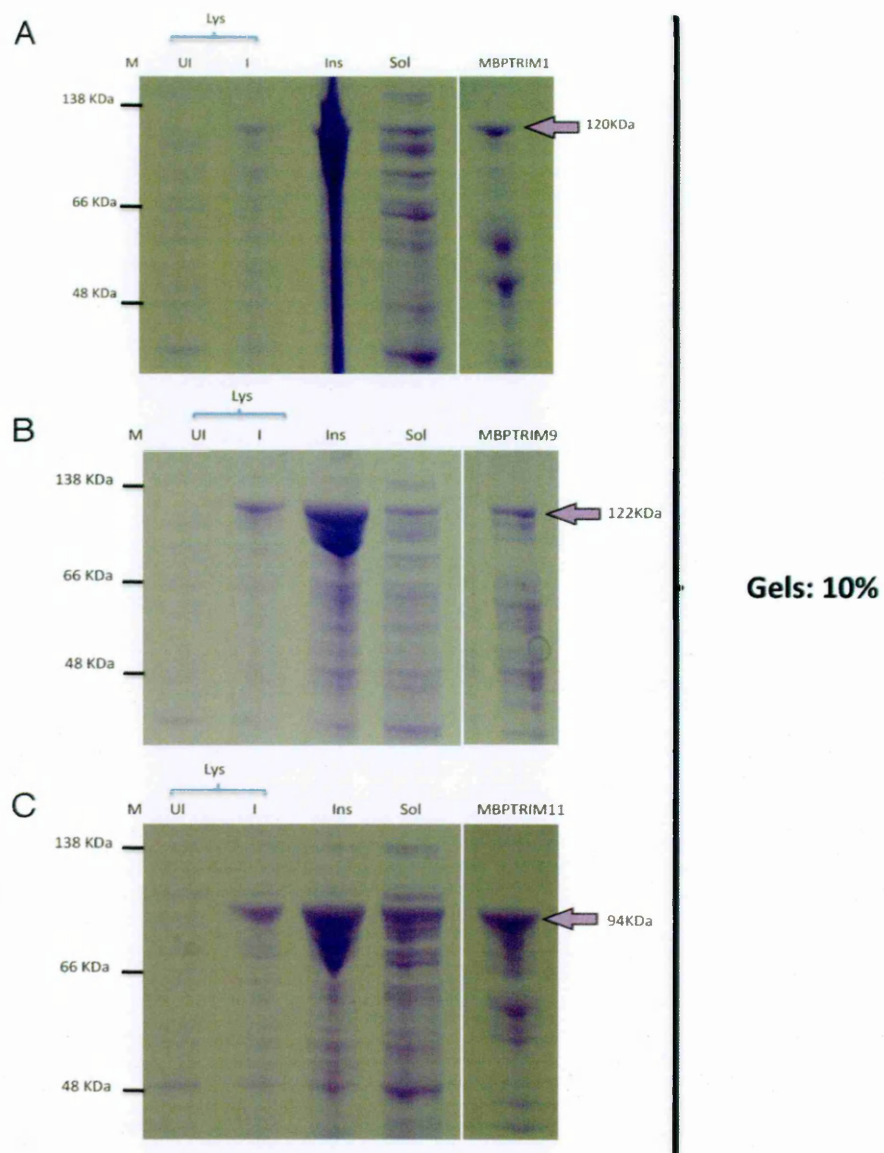
MBP pull-down uses an MBP-fusion protein (bait) bound to amylose-coupled beads to affinity purify any proteins (prey) that interact with the bait from a pool of proteins in solution. I chose to use TRIM proteins as bait and UBE2 enzymes as prey. Bait and prey proteins can be generally obtained from multiple sources including cell lysates, purified proteins, expression systems and *in vitro* transcription/translation systems. In all my experiments I tested purified MBP-TRIM fusion proteins and MycGFP tagged-UBE2 (MycGFP-UBE2) enzymes transiently transfected in HEK293.

4.2.2 Expression and purification of MBP-TRIM proteins

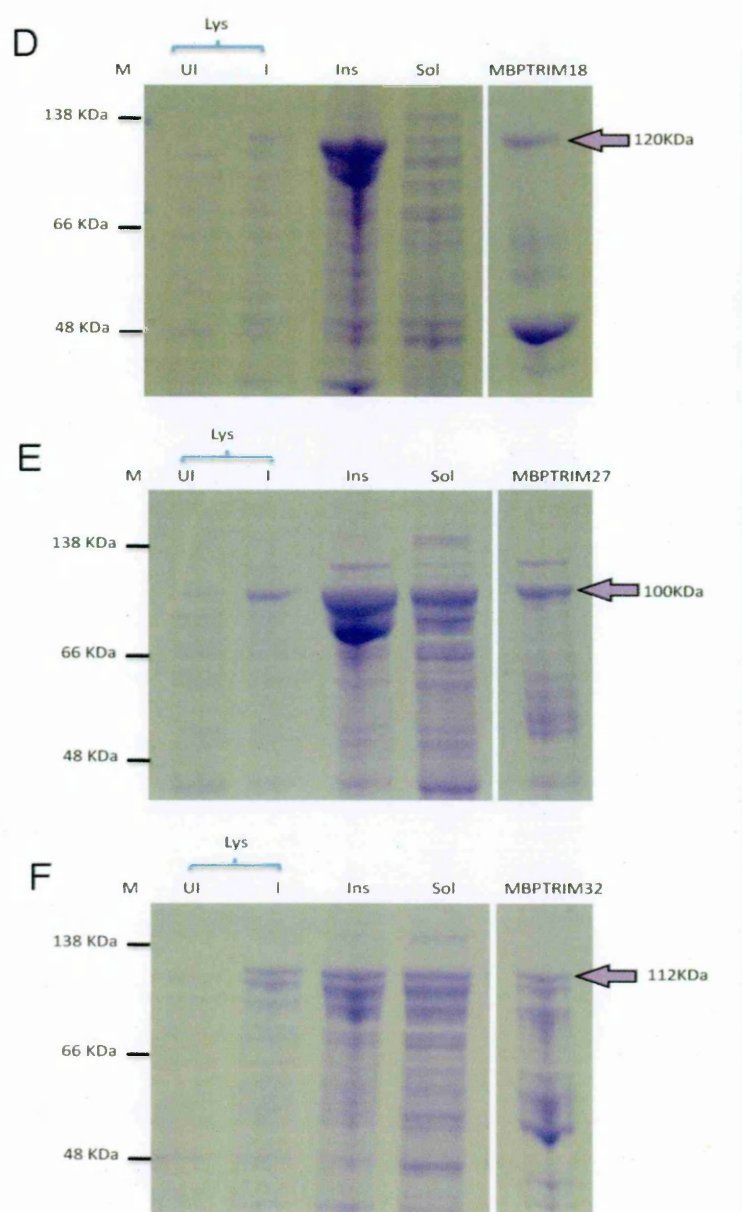
To produce MBP-TRIM proteins, I transformed the six pMAL-TRIM constructs (TRIM1, TRIM9, TRIM11, TRIM18, TRIM27, TRIM32) in *E. coli* Rosetta cells that were grown at 37 °C as indicated in paragraph 3.5. When *E. coli* cells harbouring the recombinant genes were induced with 150 μ M of IPTG for approximately 5 hours, all fusion MBP-TRIM proteins were markedly overproduced (**Figure 19**-second lane for each MBP-TRIM protein gel). Coomassie-stained gel analysis of the crude extracts indicated that the fusion proteins with the expected molecular mass accounted for 20-30% of the total cell proteins. Even if all MBP-TRIM proteins were mainly detected in the insoluble fraction (60%) than in the soluble one (40%) (**Figure 19**-third and fourth lanes MBP-TRIM protein gel), the amount and the purity of the MBP-TRIM proteins produced were sufficient for the biochemical assays performed. In all cases, improper bands may result from high-molecular-weight aggregate or truncated fragment.

The fusion proteins were further purified with the amylose resin. Coomassie-stained gel analysis of the eluted fraction revealed that procedures whereby MBP-TRIM proteins were bound to the column, washed and eluted, led to their recovery in the 60% pure form. Finally, I appropriately concentrated (2 mg/ml) all TRIM protein pure forms for further biochemical assays.

Figure 19



Coomassie gel staining of high-level expression and purification of TRIM proteins fused to MBP. (A) MBP-TRIM1. Lanes are: 1, un-induced MBP-TRIM1 (UI); 2, induced MBP-TRIM1 (I); 3: insoluble fraction of the crude extracts (Ins); 4: Soluble fractions of the crude extracts (Sol); 5: column eluted MBP-TRIM1; M: molecular weight standards in kDa. (B) MBP-TRIM9. See legend in A). (C) MBP-TRIM11. See legend in A). (D) MBP-TRIM18. See legend in A).



(D) MBP-TRIM18. See legend in A). (E) MBP-TRIM27. See legend in A). (F) MBP-TRIM32. See legend in A).

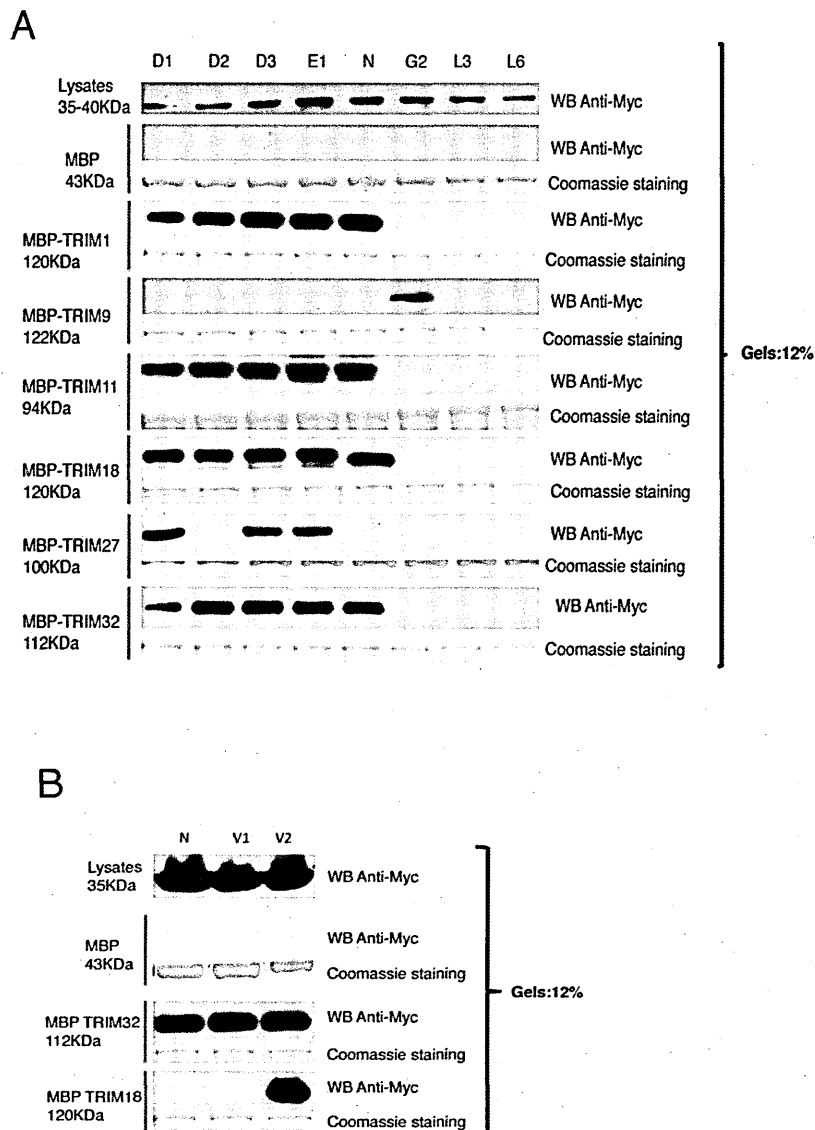
4.2.3 MBP pull-down experiments

To confirm the TRIM-UBE2 binding specificity, I used the MBP-pull down approach on a selection of TRIM and UBE2 enzymes representative of the entire spectrum of the interactions observed in the two-hybrid screening. In the assays, I chose TRIM1, 9, 11, 18, 27, 32 and 9 UBE2 enzymes (D1, D2, D3, E1, N, L3, L6, G2, V1, V2).

MBP-fusion proteins immobilized on the beads were incubated with HEK293T crude lysates transiently expressing MycGFP-UBE2 enzymes and specific binding was revealed by immunoblot (**Figure 20**). UBE2D1, D2, D3, E1 and N were captured by TRIM1, 11, 18 and 32 confirming the interactions observed in yeast. Interestingly, TRIM27 binds only UBE2D1 and D3 but not the highly similar UBE2D2 that also showed a very weak interaction in yeast. TRIM9 only bound UBE2G2 confirming its highly unique interaction. As expected, no binding could be observed with UBE2L3 and L6, reflecting the specific nature of TRIM-UBE2 interactions (**Figure 20a**). MBP-TRIM32 and MBP-TRIM18 were also incubated with lysates of HEK293T cells transiently transfected with UBE2N, V1 and V2. As also observed in yeast, TRIM32 also bound UBE2V1 and V2 in addition to UBE2N. As expected TRIM18 bound UBE2N but was unable to bind UBE2V enzymes (**Figure 20b**). I repeated the experiments three times and every time I confirmed all the results.

Thus, the specific interaction of the selected TRIM proteins with defined UBE2 enzymes observed in yeast was validated by *in vitro* binding analysis.

Figure 20



MBP pull-down assay confirms UBE2 and TRIM proteins specific interactions. A) MBP pull-down analysis of eight MycGFP-UBE2 enzymes transiently expressed in HEK293T cells (D1, D2, D3, E1, N, G2, L3, and L6 as indicated) with six MBP-TRIM proteins (TRIM1, 9, 11, 18, 27, and 32). MBP was used as control. Immunoblot with anti-Myc antibody of the input lysates is shown in the upper panel (Lysates). For each MBP-TRIM protein the anti-Myc immunoblot and the Comassie Blue staining of the gel are shown. B) MBP pull-down analysis of three MycGFP-UBE2 transiently expressed in HEK293T cells (N, V1 and V2 as indicated) with MBP-TRIM32 and MBP-TRIM18. MBP was used as control. Legend as in A). The MBP-pull down experiments were repeated three times and every time all the TRIM-UBE2 specific interactions have been confirmed as highlighted in the picture above.

4.3 *In Vitro Ubiquitylation assay*

To establish that selective UBE2 binding by TRIM proteins is translated into functional ubiquitin ligase activity, I performed *in vitro* ubiquitylation assays that test the ability of a putative E3 ligase to catalyze polyubiquitylation *in vitro* by means of different UBE2 enzymes. The *in vitro* ubiquitylation should result from mixing all the components required for the ubiquitylation reaction: ATP, ubiquitin peptide, E1 and E2 enzymes and the candidate E3 ligase (see 3.7 paragraph).

Consistently, the above 6 MBP-TRIM proteins were incubated with all ubiquitylation cascade components. The immunoblot analysis of the reaction products using antibodies against ubiquitin revealed the presence of HMW polyubiquitylation species when the recombinant MBP-TRIM protein was added to the reaction mixture (**Figure 22**). To confirm that TRIM proteins are the essential E3 in the ubiquitylation assay performed, for each TRIM protein tested I set up incomplete mixtures containing different combinations of the reagents (ATP, Ubiquitin, E1 enzyme, E2 enzyme and TRIM protein). The immunoblot analysis of the reaction products using both antibodies against ubiquitin (**Figure 21-upper panel**) and antibodies against the MBP-tag highlighted (**Figure 21-lower panel**) that lack of MBP-TRIM proteins as well as the above reagents did not result in the detection of the polyubiquitylation smear. This underlies that E1 enzyme, UBE2 enzyme and TRIM proteins are all required for *in vitro* ubiquitylation reaction.

Concomitantly, I also addressed the specific UBE2 usage in these reactions. The *in vitro* ubiquitylation assays were carried out in the presence of one of the following recombinant enzymes as UBE2: D1, D2, D3, E1, N, J2, G2, L3, L6. Figure 21a shows that incubation of MBP-TRIM1, 11, 18 and 32 in the presence of either UBE2D1, D2, D3, E1, or N resulted in polyubiquitylation recapitulating the binding specificity observed in yeast and in MBP pull-down assays. Moreover, MBP-TRIM11 acted as E3 also in the presence of UBE2J2 that was not present in the original two-hybrid panel. As

expected from the binding data, MBP-TRIM27 displayed specific E3 activity in the presence of UBE2D1 and D3 but not with the non-interacting D2. The remarkable binding specificity of MBP-TRIM9 for UBE2G2 was also manifest in the functional assay where E3 ligase activity was only observed when TRIM9 was incubated in the presence of UBE2G2. Consistently with the specific data observed through the Interaction Mating screening, the incubation of MBP-TRIM proteins in the reaction mixture containing UBE2L3 and L6 did not result in the formation of any polyubiquitylated species (**Figure 22a**).

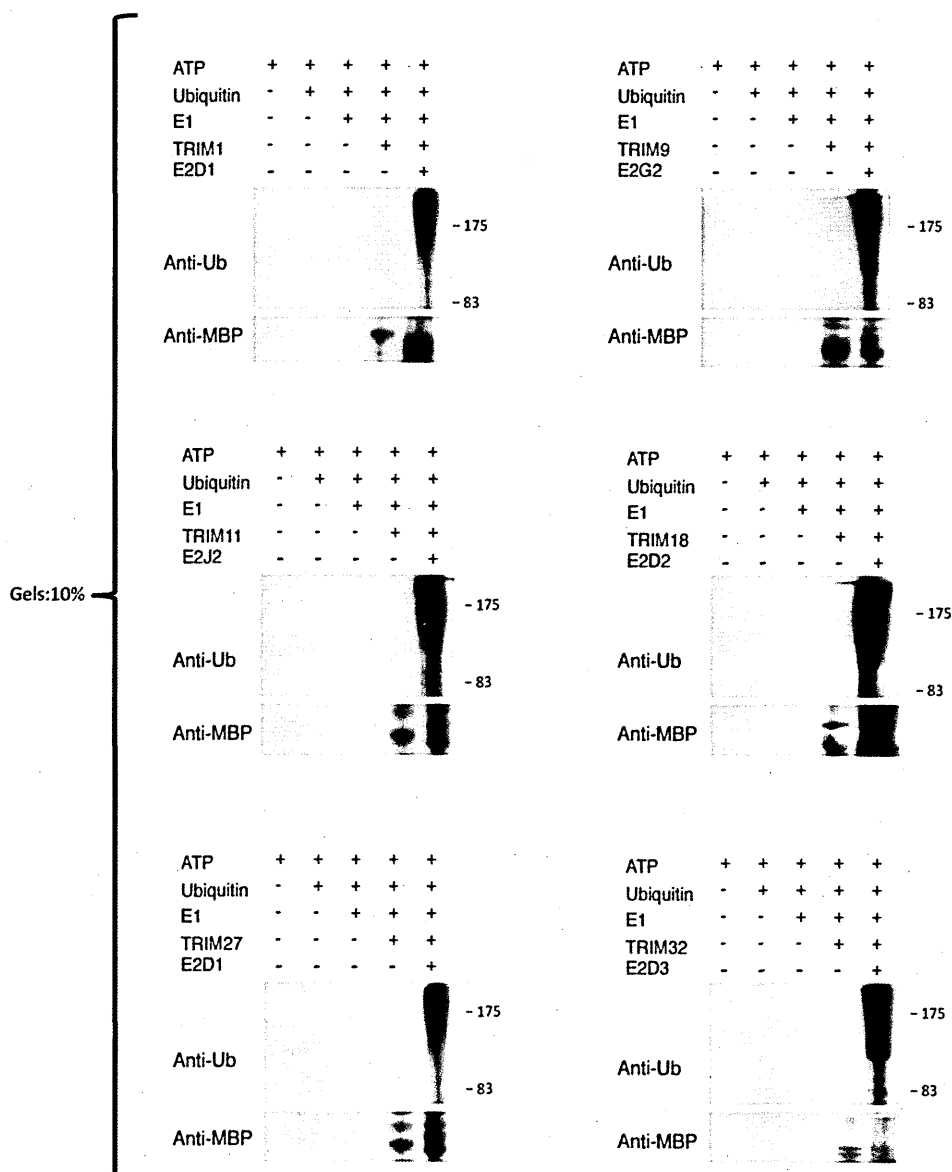
As both yeast two-hybrid and MBP-pull down assays highlighted the interaction between TRIM32 and UBE2V, I tested TRIM32 polyubiquitylation in the presence of ATP, recombinant E1 enzyme, ubiquitin, recombinant UBE2N and V1 proteins. It is known that V1 is a non-autonomous UBE2 that lacks the catalytic cysteine (Cys) and can only act in cooperation with UBE2N. Consistently, TRIM32 was a more efficient E3 in the presence of both UBE2N and V1 than with UBE2N alone (**Figure 22b - left panel**).

I also analyzed the reactions with antibodies against the MBP portion of the protein, which showed that in the majority of the cases polyubiquitylated species are mainly represented by the MBP-TRIM proteins themselves (**Figure 23b**). The results with MBP-TRIM1, MBP-TRIM11, MBP-TRIM18, MBP-TRIM32 perfectly matched the results obtained with the anti-ubiquitin antibody (**Figure 23b and Figure 23c**). This is not the case with MBP-TRIM9 and MBP-TRIM27 where I detected a reduced self-polyubiquitylation. It is possible that TRIM9 and TRIM27 might ubiquitylate other proteins present in the reaction mixture (E1, UBE2 or *E. coli* proteins) more efficiently than they ubiquitylate themselves.

Taken together, these results indicate that TRIM proteins act as E3 ligases cooperating with the ubiquitylation machinery in a very specific manner that

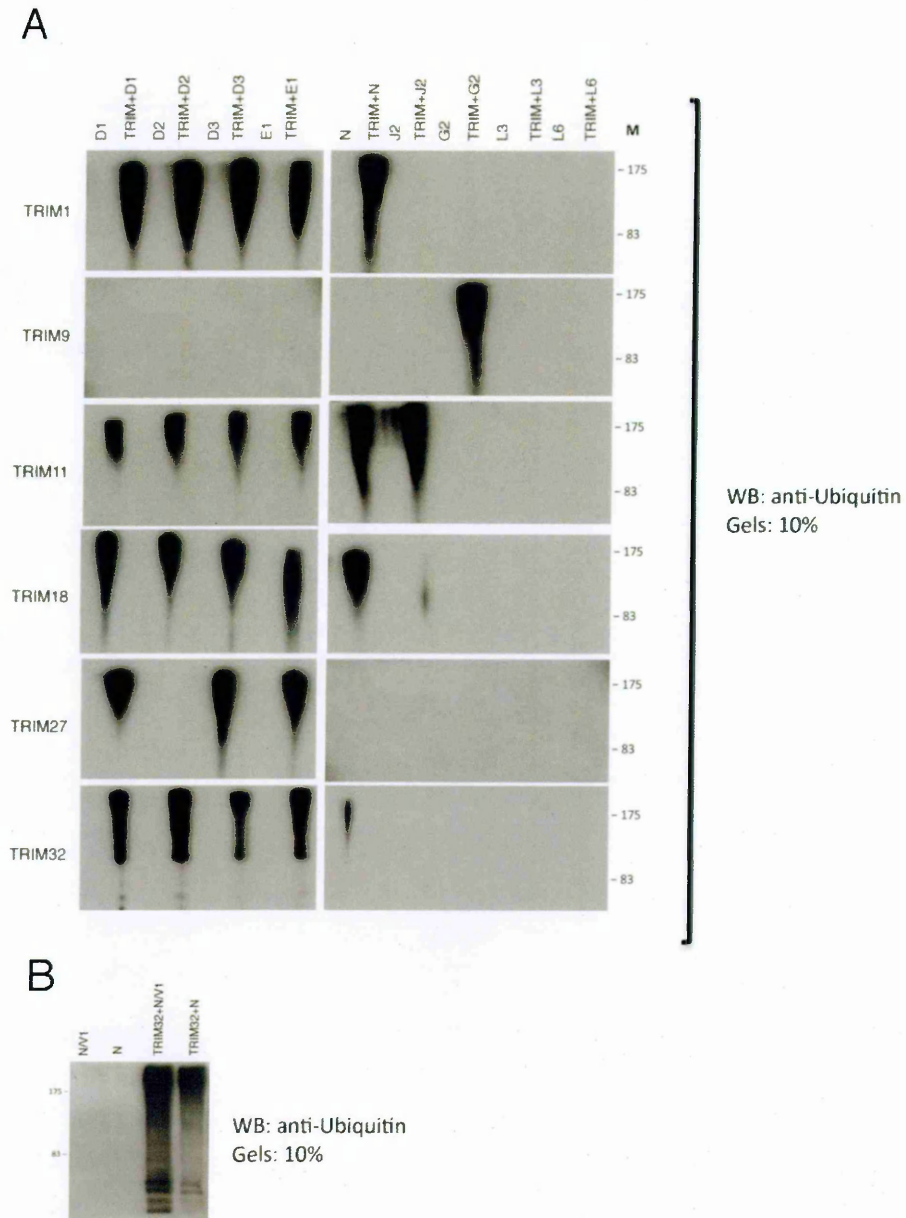
recapitulates the specific TRIM-UBE2 interactions observed both in Interaction Mating screening and by MBP-pull down assay.

Figure 21



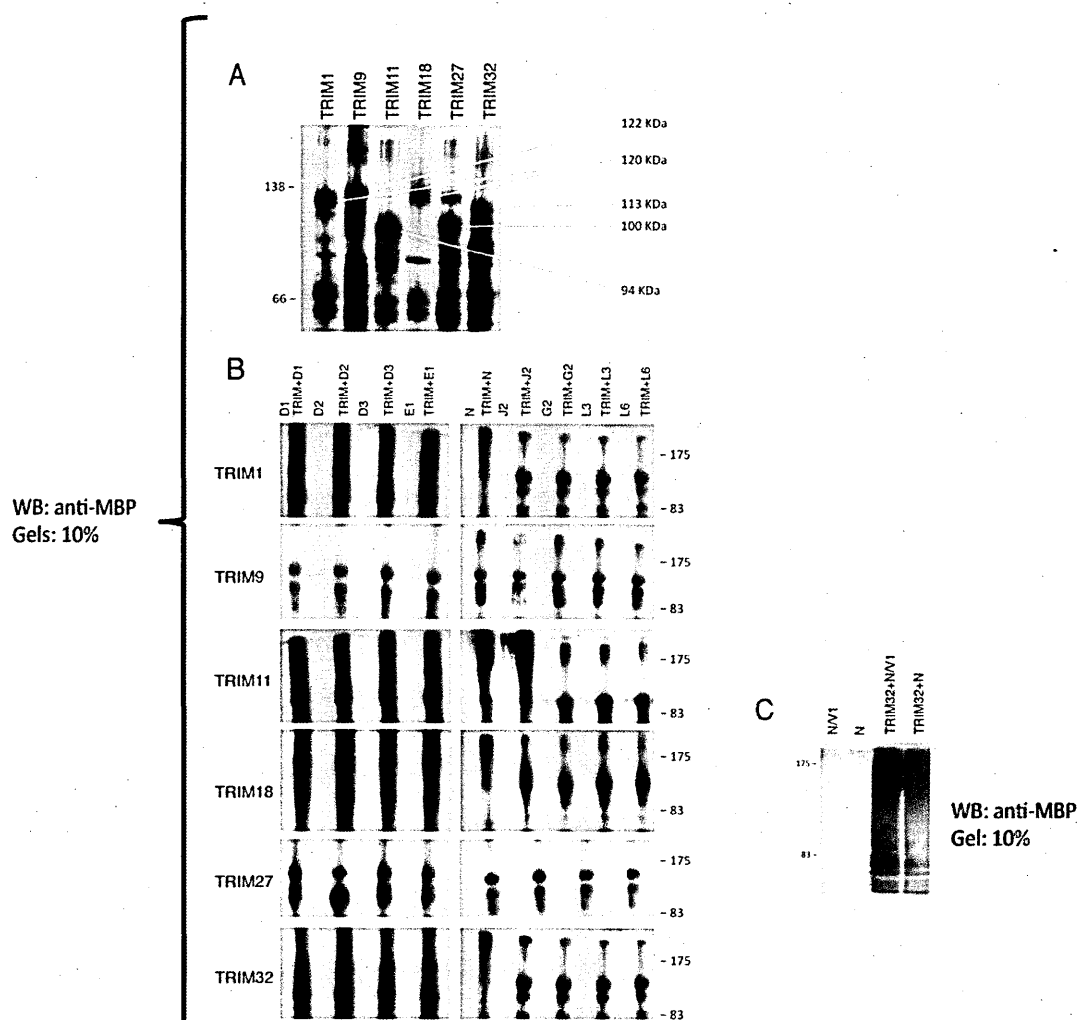
Control of the *in vitro* ubiquitylation assay. To confirm that TRIM proteins are the essential E3 in the ubiquitylation reactions, for each TRIM protein tested I set up incomplete mixtures containing different combinations of the reagents (ATP, Ubiquitin, E1 enzyme, E2 enzyme and TRIM protein) as indicated in the figure. The reactions were repeated four times for each TRIM protein selected and analyzed by immunoblotting using anti-Ubiquitin (upper panel) and anti-MBP (lower panel) antibodies. Lack of TRIM proteins as well as of any other of the above reagents did not result in the detection of poly-ubiquitylation smear (lane 1-4). As a positive control, ubiquitylation was observed with the incubation of MBP-TRIM proteins with the complete mixture (lane 5). The picture reported above represents the results obtained in all four experiments.

Figure 22



TRIM proteins act as ubiquitin E3 ligases *in vitro* using the interacting UBE2 enzymes. A) MBP-TRIM proteins (TRIM1, 9, 11, 18, 27 and 32) were tested for E3 ligase activity in *in vitro* ubiquitylation assay in the presence of the UBE2 enzymes indicated (TRIM+D1, D2, D3, E1, N, G2, J2, L3, L6). As control, the assay was performed without the TRIM protein (lanes with only UBE2 indicated). Immunoblot with anti-Ubiquitin antibody to detect the ubiquitylated species is shown. B) *In vitro* ubiquitylation assay using UBE2N and V1 in the combination indicated in the presence of MBP-TRIM32. *In vitro* Ubiquitylation assays were repeated four times for all the TRIM proteins tested in the same order reported in the picture above that is representative of all the four experiments. Immunoblot with anti-ubiquitin antibody is shown. M, molecular weight marker.

Figure 23



Immunoblots using anti-MBP antibody. A) Anti-MBP immunoblot of the purified MBP-TRIM fusion proteins. The full-length MBP-TRIM proteins and their molecular weight are indicated. B) Anti-MBP immunoblot of the *in vitro* ubiquitylation reactions described in the text and revealing the extent of self-ubiquitylation. The results with MBP-TRIM1, MBP-TRIM11, MBP-TRIM18 and MBP-TRIM32 perfectly matched what observed with the anti-ubiquitin antibody (Figure 4M) indicating that self-ubiquitylation is mainly occurring. This is not the case with MBP-TRIM9 and MBP-TRIM27 where I detected reduced self-polyubiquitylation. C) Anti-MBP immunoblot of the ubiquitylation reactions of TRIM32 with the UBE2N/V1 complex. All the *In vitro* Ubiquitylation assays shown were repeated four times for all the TRIM proteins tested in the same order reported in the picture above that is representative of all the four experiments. Immunoblot with anti-ubiquitin antibody is shown. M, molecular weight marker.

4.4 *In vivo Ubiquitylation assay*

Since *in vitro* ubiquitylation assays determined the E3 activity of the TRIM proteins tested, this raises the question of whether TRIM proteins could act as E3 ligases in mammalian cells. To evaluate TRIMs' ability to induce self-ubiquitylation in mammalian cells, I performed *in vivo* ubiquitylation assays using the six selected TRIM proteins both as full-length and as UBE2 binding incompetent RING deleted forms. Indeed, as already observed for *in vitro* ubiquitylation assays, self-ubiquitylation is an important determinant to define an E3 ubiquitin ligase. Usually, candidate E3 ubiquitin ligase is co-transfected together with ubiquitin in mammalian cells. Then, the E3 is immunoprecipitated with a specific antibody and immunoblotting performed with the anti-ubiquitin antibody shows the ubiquitin chain(s) attached to the E3.

MycGFP-tagged wild-type TRIM1, 9, 11, 18, 27, 32 and their RING deletion mutants were co-transfected in HEK293T cells together with HA-tagged ubiquitin. TRIM proteins were immunoprecipitated with the anti-Myc antibody and analyzed by western blotting using either an antibody against the HA tag to detect ubiquitylated proteins (**Figure 24a, upper panels**) or an antibody against the Myc tag to check for total immunoprecipitated MycGFP-TRIM proteins (**Figure 24a, lower panels**). Immunoblotting using anti-HA showed a high molecular weight (HMW) smear for each TRIM protein tested, suggesting that they are self-polyubiquitylated and/or that proteins co-immunoprecipitating with them are polyubiquitylated in mammalian cells (**Figure 24a, left upper panels**). Interestingly, mainly in the case of TRIM1, immunoblot with anti-HA reveals the presence of a strong single band at expected MycGFP-TRIM1 molecular weight. This could highlight an exclusive TRIM1 monoubiquitylation in HEK293T cells.

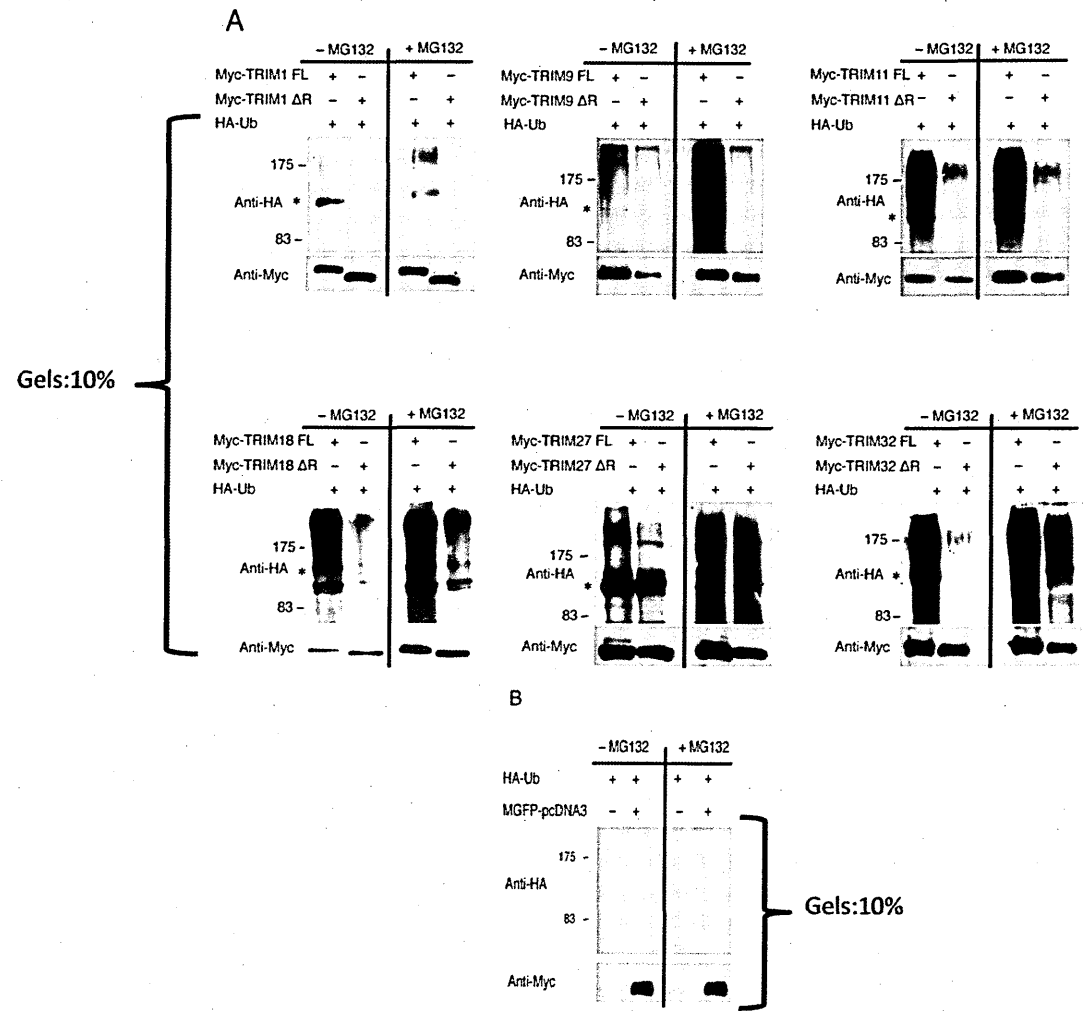
In contrast, the ubiquitylation observed in the presence of the corresponding RING-deleted mutant was extremely weak suggesting that the HMW species were likely produced mainly through the TRIM protein E3

activity (**Figure 24a, left upper panels**). The residual faint ubiquitylation observed in correspondence of the RING-deleted TRIM protein might be due to the activity of the endogenous TRIM protein or of other E3 ligases. This still undefined endogenous E3 activity appears to be more relevant in the case of TRIM27 where even in the presence of the RING-deleted form a comparable polyubiquitylation was observed. Moreover, HEK293T cells transfected with HA-ubiquitin, either alone or with MycGFP-empty vector, produced no ubiquitylated species upon anti-Myc immunoprecipitation (**Figure 24b**).

To determine whether the observed *in vivo* ubiquitylation was mainly associated to proteasome-mediated degradation, the experiments described above were also performed in the presence of the proteasomal inhibitor MG132. Treatment with MG132 increased both the ubiquitylation (**Figure 24a, right upper panels**) and the total amount of the TRIM proteins tested (**Figure 24a, right lower panels**). Interestingly, this treatment clearly increased also the ubiquitylation of TRIM27 and TRIM32 RING deletion mutants suggesting that other E3 ligases are implicated in their ubiquitylation.

Thus, these results indicate that, although to different extent, the TRIM proteins act as RING-dependent ubiquitin E3 ligase in mammalian cells.

Figure 24



E3 ligase activity of TRIM proteins in HEK293 cells. A) *In vivo* ubiquitylation assay of MycGFP(MGFP)-tagged TRIM1, TRIM9, TRIM11, TRIM18, TRIM27 and TRIM32 full-length (FL) and RING deleted (ΔR) forms in HEK293T cells in the presence of HA-ubiquitin (HA-Ub) as indicated. Immunoblot of the anti-Myc immunoprecipitates using anti-HA antibody to detect ubiquitylated proteins (upper panels) and anti-Myc antibody to detect TRIM proteins (lower panels) are shown. Where indicated, cells were treated with the MG132 proteasomal inhibitor for six hours. Asterisks indicate expected TRIM proteins molecular weights. B) HEK293T cells transfected with HA-Ubiquitin either alone or together with MycGFP-pcDNA3 vector were immunoprecipitated with anti-Myc antibody. Legend as in A). All the *In vivo* Ubiquitylation assays were repeated three times for each TRIM protein tested. The picture reported above is representative of all the three experiments repeated for every TRIM protein selected.

4.5 Specific TRIM-UBE2 binding occurs in HeLa cells

How selection and usage of UBE2 enzymes is achieved and what are the consequences on TRIM activity within the cellular context is a more complicated issue to address. *In vivo* selection may depend on a variety of parameters including the spatial accessibility of the E2 enzyme.

To address this issue, the subcellular distribution of the TRIM proteins and their interacting UBE2 enzymes was investigated by immunofluorescence after transfection of GFP- and HA-tagged constructs in HeLa cells. As already reported, TRIM1, 9, 11, 18, 27, and 32 were mainly localized in the cytoplasm of HeLa cells either in filamentous or speckled structures (Reymond, Meroni et al. 2001). UBE2D1, D2, D3, G2, N, V1, and V2 were diffused in both nucleus and cytoplasm (**Figure 25a** and **Figure 26a**). The only exception was UBE2E1, which is a strictly nuclear protein (Plafker, Plafker et al. 2004). When co-transfected with TRIM proteins, the UBE2 enzymes generally maintained their distribution and were not apparently enriched in the TRIM defined cellular structures consistent with the fact that several of them are shared by many E3 ligases.

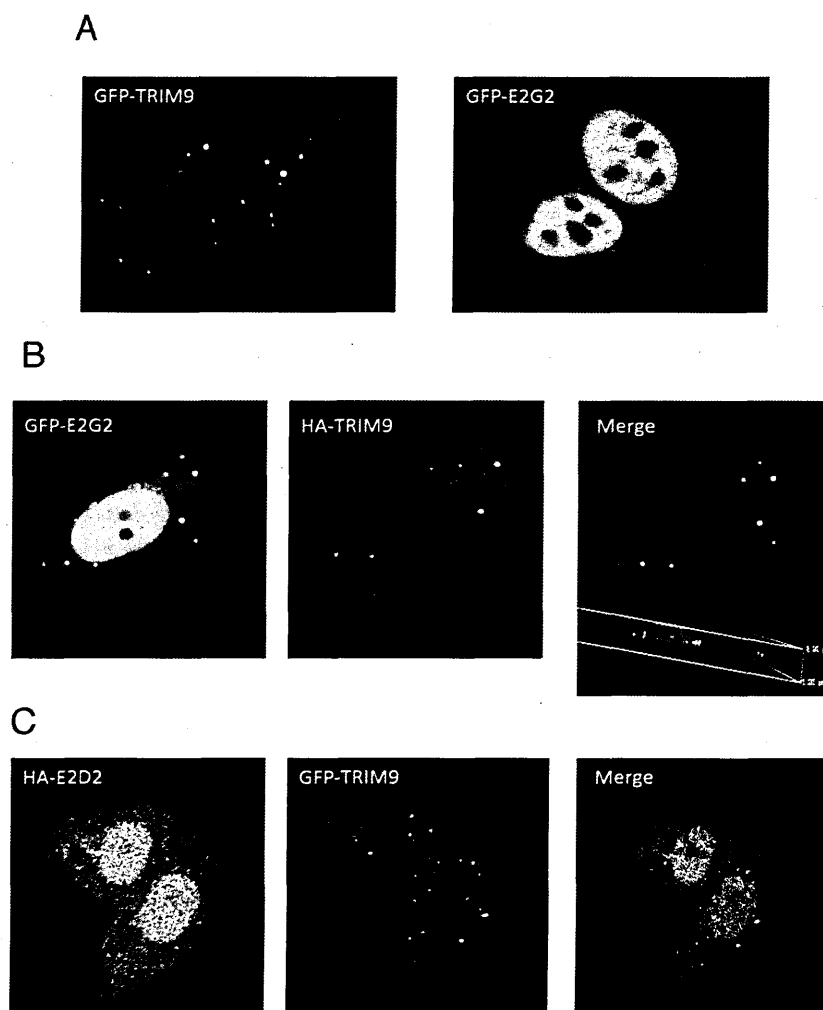
However, one exception was observed with the UBE2G2 and TRIM9 partnership. As already shown (Reymond, Meroni et al. 2001), TRIM9 is present in cytoplasmic speckles while GFP-UBE2G2 is diffused in both the nucleus and the cytoplasm (**Figure 25a**). When co-transfected with TRIM9 in HeLa cells, a fraction of UBE2G2 was clearly recruited into TRIM9 cytoplasmic speckles as also demonstrated by a collection of multiple focal planes (z-stack) (**Figure 25b**). Consistent with TRIM-UBE2 interaction data, when HeLa cells were co-transfected with HA-TRIM9 and GFP-UBE2D2, the latter conserved its diffuse distribution in the cell and it was not recruited by exogenous TRIM9, supporting the specific effect on UBE2G2 (**Figure 25c**). To clarify if other TRIM proteins were able to exert the same effect on UBE2G2, I also transfected HeLa cells with GFP-UBE2G2 and either HA-tagged TRIM1, TRIM11, TRIM18, TRIM27, or TRIM32. UBE2G2 was not

recruited by TRIM1, TRIM11, TRIM18 and TRIM32 into any defined structures further supporting the specificity of what observed with TRIM9. Interestingly, even if TRIM27 formed cytoplasmic speckles like TRIM9, the UBE2G2 protein was never found within these structures (**Figure 25d**).

Moreover, I also observed that TRIM32 changed its own localization when co-transfected with either UBE2N or UBE2V2 proteins. In agreement with the literature (Reymond, Meroni et al. 2001), TRIM32 was localized in cytoplasmic perinuclear speckles, whereas UBE2N and UBE2V proteins were diffused within the cytoplasm and the nucleus (**Figure 26a**). When co-transfected with GFP-UBE2N and HA-TRIM32, 40% of HeLa cells showed a perfect co-localization of TRIM32 and UBE2N in the nucleus (**Figure 26b, upper panels**). In 60% of the cells I observed TRIM32 localized in well-defined speckles around the nucleus whereas UBE2N was diffused in both the nucleus and the cytoplasm (**Figure 26b, lower panels**). The peculiar TRIM32-UBE2N reciprocal localization was not observed when HeLa cells were co-transfected with GFP-UBE2N and HA-TRIM18 that we previously found to interact (**Figure 26f**). When we transfected HeLa cells with HA-TRIM32 and GFP-UBE2V1, the latter was partially recruited in TRIM32 speckles around the nucleus (**Figure 26c**). Curiously, although few cells were co-transfected with HA-TRIM32 and GFP-UBE2V2 (approximately 30%), I found a complete co-localization of the two in a diffused UBE2V2-like manner (**Figure 26d**). Consistently, the specific TRIM32/UBE2N and TRIM32/UBE2V2 localization was observed also when I transfected HeLa cells with GFP-TRIM32 and HA-UBE2N/UBE2V2 (**Figure 26e**).

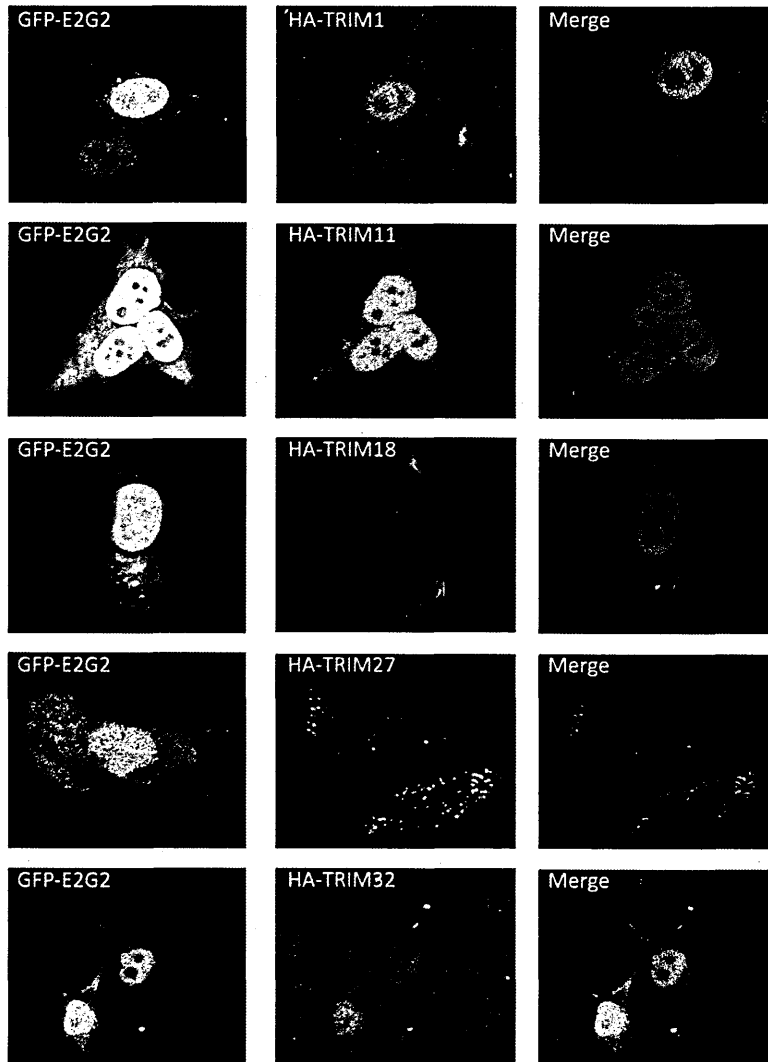
Thus, immunofluorescence experiments indicate that binding of several E2-TRIM pairs very likely occurs also *in vivo*.

Figure 25



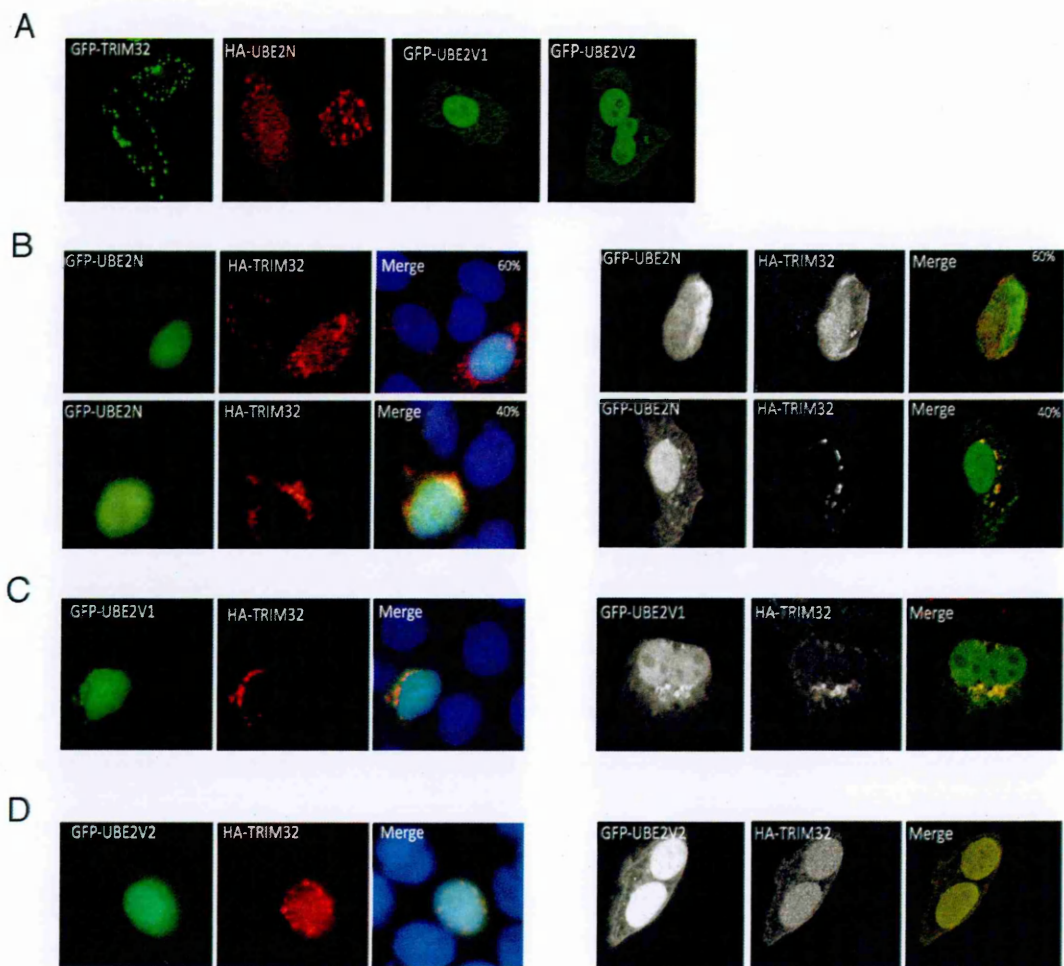
TRIM9 co-localizes with UBE2G2 in cytoplasmic speckles. A) Immunofluorescence of HeLa cells transfected with either GFP-TRIM9 (left panel) or GFP-UBE2G2 (right panel). **B)** Immunofluorescence of HeLa cells co-transfected with GFP-UBE2G2 (left panel) and HA-TRIM9 (middle panel). The right panel represents the overlay of left and middle images. The inset shows a lateral view of the above cell as resulting from a z-stack collection of confocal images.

D



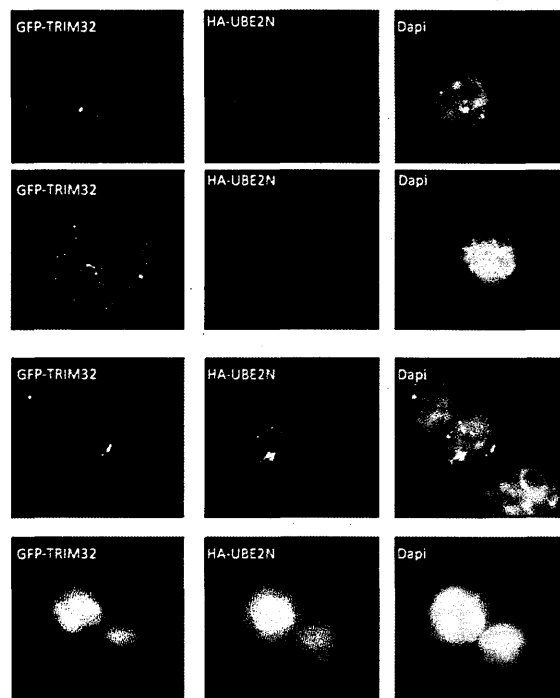
UBE2G2 does not colocalize with TRIM1, TRIM11, TRIM18, TRIM27 and TRIM32. Immunofluorescence of HeLa cells co-transfected with GFP-UBE2G2 (left panels) and HA-TRIM1, HA-TRIM11, HA-TRIM18, HA-TRIM27, HA-TRIM32 (middle panels). The right panels represent the overlay of left and middle image

Figure 26

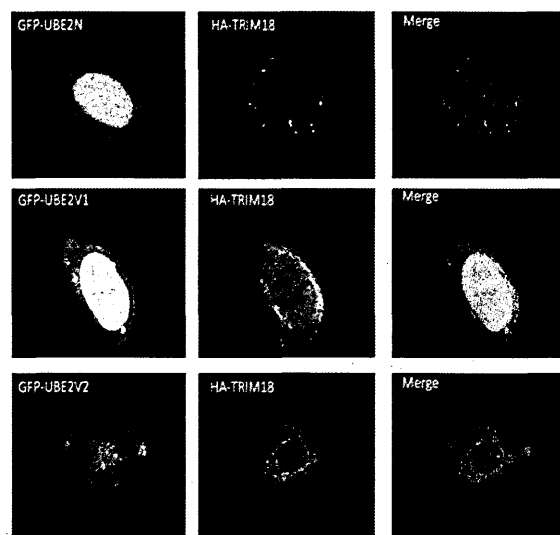


TRIM32 co-localize with UBE2N, V1, V2 proteins. A) Immunofluorescence of HeLa cells single transfections with: GFP-TRIM32, HA-UBE2N, GFP-UBE2V1, GFP-UBE2V2. **B)** Immunofluorescence of HeLa cells co-transfected with GFP-UBE2N (left panels) and HA-TRIM32 (middle panels). The right panels represent the overlay of left and middle images. Two behaviours of TRIM32 in the presence of UBE2N are represented and their percentage shown (40% in the upper panel; 60% in the lower panel). **C)** Immunofluorescence of HeLa cells co-transfected with HA-TRIM32 (left panel) and GFP-UBE2V1 (middle panel). The right panels represent the overlay of left and middle images. **D)** Immunofluorescence of HeLa cells co-transfected with HA-TRIM32 and GFP-UBE2V2. Legend as in C).

E



F



E) The same experiment shown in Figure 4Q(b)-4Q(d) of the main text performed with the reciprocally tagged constructs as indicated (GFP-TRIM32 and HA-UBE2N, V1 and V2). **F)** The same experiment shown in Figure 4Q(b)-4Q(d) of the main text using GFP-UBE2N and HA-TRIM18.

4.6 TRIM proteins involvement in SUMOylation pathway.

4.6.1 TRIM proteins interaction with UBE2I

To further investigate TRIM proteins involvement in SUMOylation pathway, I started to test interaction between TRIM proteins and UBE2I, which is the specific SUMO UBE2 enzyme (see Introduction), through the Interaction Mating technique. Then, as already shown in paragraph 4.2, I investigated the interaction between a sub-group of the TRIM protein family and the UBE2I enzyme through MBP-pull down assay. It is well known the involvement of TRIM19/PML in the SUMOylation pathway that has a key role in the organization of PML-Nuclear Bodies (Zhong, Muller et al. 2000). Indeed, recently Chu and Yang (2010) demonstrated that some TRIM family members can efficiently interact with UBE2I and strongly enhance transfer SUMO 1-3 from UBE2I to Mdm2, p53 E3 Ubiquitin Ligase (Chu and Yang 2011).

1. Interaction Mating assays

I tested the 42 TRIM proteins listed in **TABLE 3** expressed both as bait and as prey in the yeast EGY48 (mating type α) and in the yeast strain EGY42 (mating type a) against UBE2I protein expressed in both vectors and yeast strains. To be certain of the interactions observed, I repeated the Interaction Mating assays seven times and every time, before performing the Interaction Mating experiments, I freshly transformed TRIM and UBE2I pEG202 and pJG4-5 constructs in yeast strain (see paragraph 3.2.1). I summarized the results of the Interaction Mating assays in **TABLE 4**.

These data revealed that the majority of the TRIM proteins tested interact with UBE2I enzyme suggesting a putative involvement of the TRIM

proteins in the SUMOylation pathway either as SUMO E3 Ligases or as SUMO substrates, or both.

Contrary to that demonstrated for TRIM/UBE2 interactions (see paragraph 4.1.4), interaction was observed also between the “unorthodox” TRIM members that lack the RING domain (Sardiello, Cairo et al. 2008) and the UBE2I enzyme, with TRIM44 being the only exception (**Figure 27b**). Consistently, Interaction Mating assays performed with TRIM18 and TRIM32 mutant constructs lacking the RING domain and UBE2I, maintained interaction between the TRIM proteins and the UBE2I enzymes (**Figure 27a**). These data confirmed the recent report demonstrating that both RING and B-box domain are necessary for TRIM19/PML to act as E3 SUMO Ligase (Chu and Yang 2011).

This indicates that in some instances the RING domain is not necessary for the binding of TRIM proteins to the UBE2I enzyme.

2. MBP pull-down assays

Then, I verified the interactions between the six above selected TRIM proteins (TRIM1, TRIM9, TRIM11, TRIM18, TRIM27, TRIM32) and UBE2I enzyme through MBP pull-down assays.

MBP-TRIM fusion proteins immobilized on the beads were incubated with HEK293T crude lysates transiently expressing MycGFP-UBE2I and specific binding was revealed by immunoblot (**Figure 28a**). MBP-pull down assays showed that all the TRIM proteins tested captured UBE2I, confirming the interactions observed in yeast, albeit with different binding affinities. Although the MBP pull-down technique is not able to determine the kinetic and energetic parameters governing the interactions between the proteins tested, TRIM27 and TRIM32 showed stronger binding to UBE2I (**Figure 28a**), whereas TRIM9 showed a weaker one (**Figure 28a**). As expected, no binding was observed between MBP protein and UBE2I enzyme.

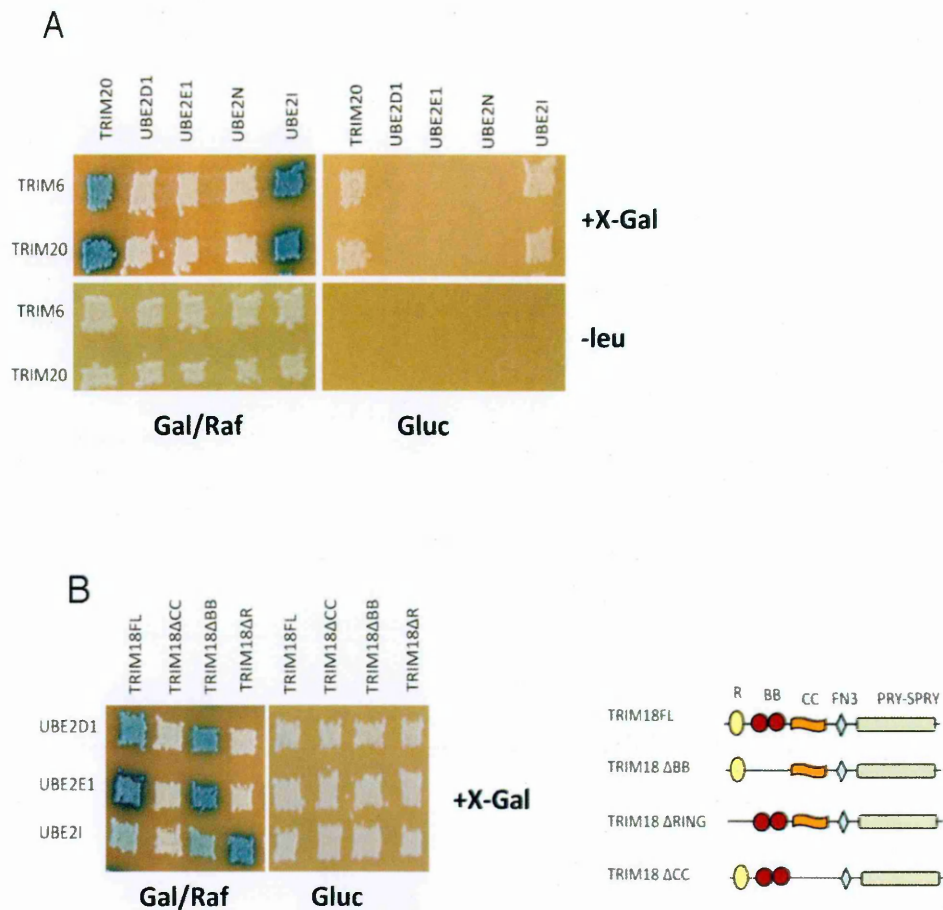
Thus, taken together these data confirmed the interactions observed in yeast Interaction Mating between the selected TRIM proteins and UBE2I with an apparent different binding. Moreover, these data have to be considered preliminary and they will require validation by other techniques.

TABLE 4

	Homo-interaction	UBE2I
TRIM1	X	X
TRIM2	X	
TRIM3	X	X
TRIM4	X	
TRIM5	X	X
TRIM6	X	X
TRIM7	X	X
TRIM8	X	X
TRIM9	X	X
TRIM10	X	X
TRIM11	X	X
TRIM12	X	X
TRIM13	X	
TRIM14*	X	X
TRIM15	X	
TRIM16*	X	X
TRIM17	X	X
TRIM18	X	X
TRIM19	X	X
TRIM20*	X	X
TRIM21	X	X
TRIM22		
TRIM23	X	X
TRIM24		
TRIM25		
TRIM26		
TRIM27	X	X
TRIM28		
TRIM29*	X	X
TRIM30	X	X
TRIM31	X	
TRIM32	X	X
TRIM37	X	X
TRIM39	X	X
TRIM40		
TRIM42		
TRIM43		
TRIM44*	X	
TRIM45	X	
TRIM46		
TRIM48		
TRIM50	X	X
TRIM52	X	

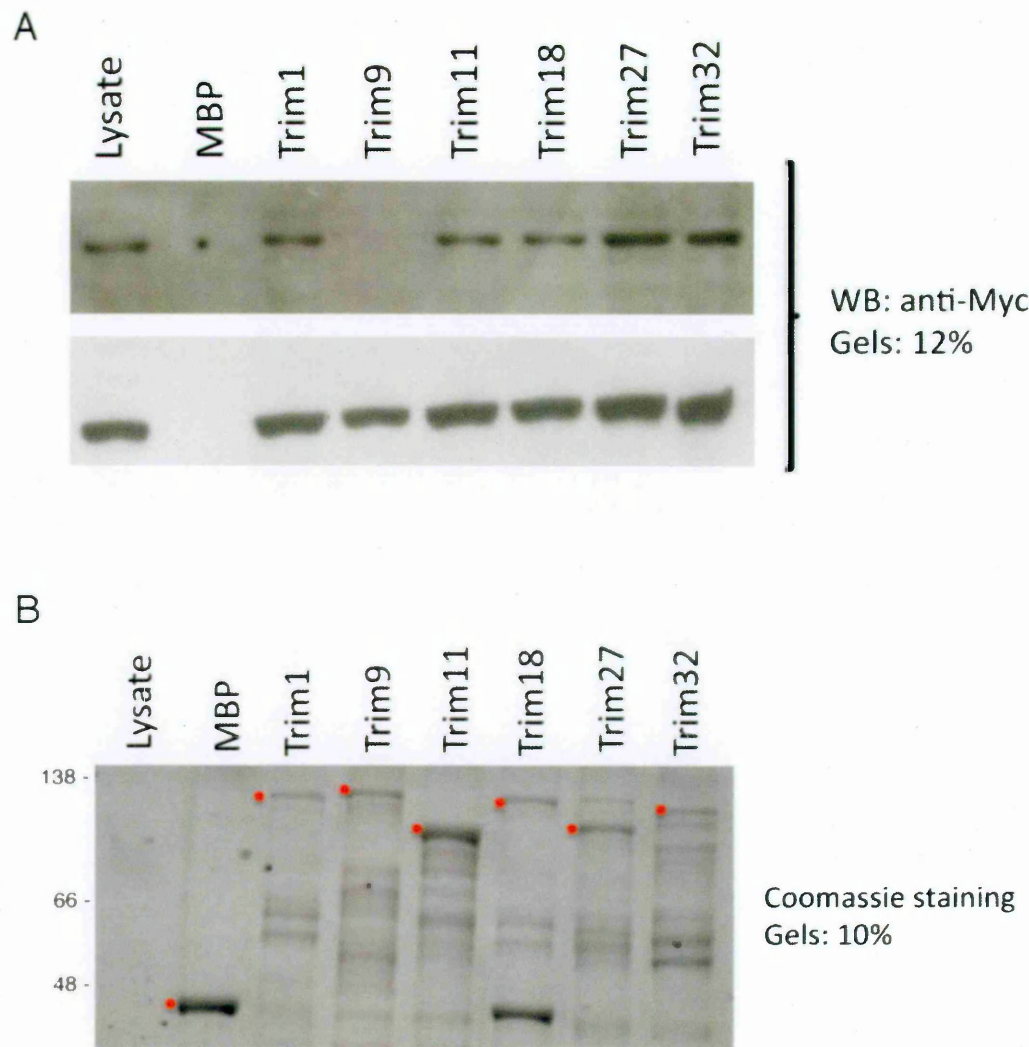
TRIM proteins interact with **UBE2I** enzyme. List of the results obtained with the binary two- hybrid system. The **TRIM** and the **UBE2I** are indicated. The asterisk and the red colour indicate the RING-less **TRIM** proteins. **TRIM** proteins homo-interaction is used as internal control.

Figure 27



Two Interaction Mating panels showing either RING-less TRIM20 (A) or TRIM18 domain deleted mutants (B), shown in the right scheme, against the UBE2 enzymes indicated. Blue colonies in X-Gal plates (+X-Gal) and growth on plates lacking leucine (-leu) in the presence of Galactose/Raffinose (Gal/Raf) represent positive interactions. The Interaction Mating screening between TRIM proteins and UBE2I was repeated seven times. The picture is representative of all the interactions observed (for details see text).

Figure 28



MBP-pull down assays confirm the interactions observed between TRIM proteins and UBE2I enzyme.

A) MBP-pull down analysis of MycGFP-UBE2I transiently expressed in HEK293T cells with six MBP-TRIM proteins (TRIM1, TRIM9, TRIM11, TRIM18, TRIM27, TRIM32). MBP was used as control. Immunoblot with the anti-Myc antibody of 1/5 of the input lysates is shown in the upper panel; immunoblot with the anti-Myc antibody of 1/2 of the input lysates is shown in the lower panel. B) Coomassie Blue staining gels of the MBP-TRIM proteins linked to amylose beads are shown. Red asterisks indicate MBP and MBP-TRIM proteins molecular weight. This experiment was repeated three times in the same order reported in the picture above that is representative of the results obtained.

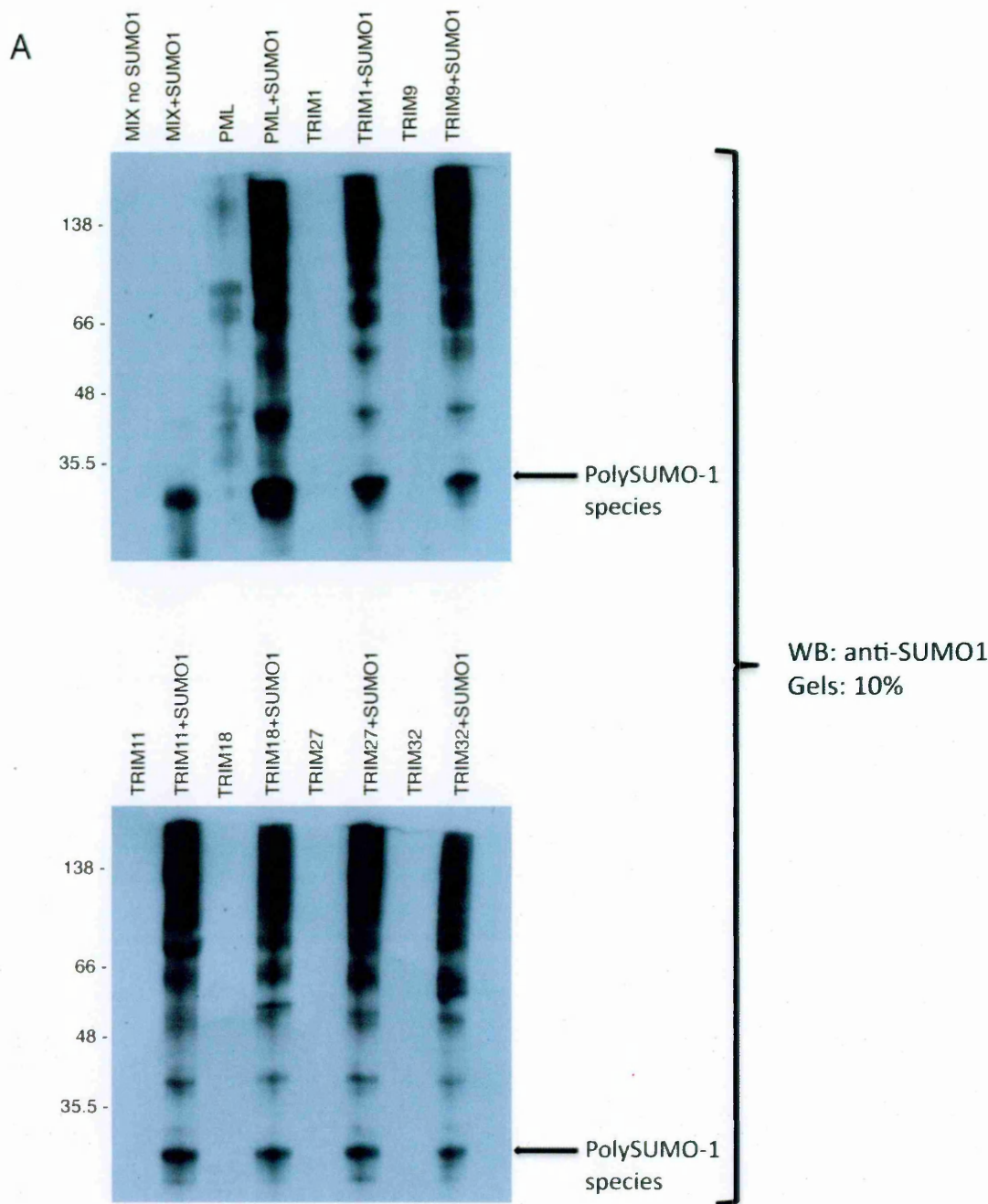
4.6.2 *In vitro* SUMOylation assays

TRIM interaction with UBE2I raised the question of whether TRIM proteins could be also modified by SUMO peptides. To address this issue, I performed *in vitro* SUMOylation assays. SUMOylation reaction is performed by mixing all SUMOylation pathway components: ATP, recombinant E1 (SAE1/2), recombinant E2 (UBE2I), the putative substrate and either SUMO1 or SUMO2. The most important difference between an *in vitro* ubiquitylation assay and an *in vitro* SUMOylation assay is that the former reveals the role as E3 Ubiquitin Ligase of the protein tested thanks to its ability to form poly-ubiquitylation species when added to the reaction mixture; the latter mainly highlights the possibility of the protein tested to be SUMOylated since the UBE2I enzyme can attach poly-SUMO chains to a substrate (Bernier-Villamor, Sampson et al. 2002).

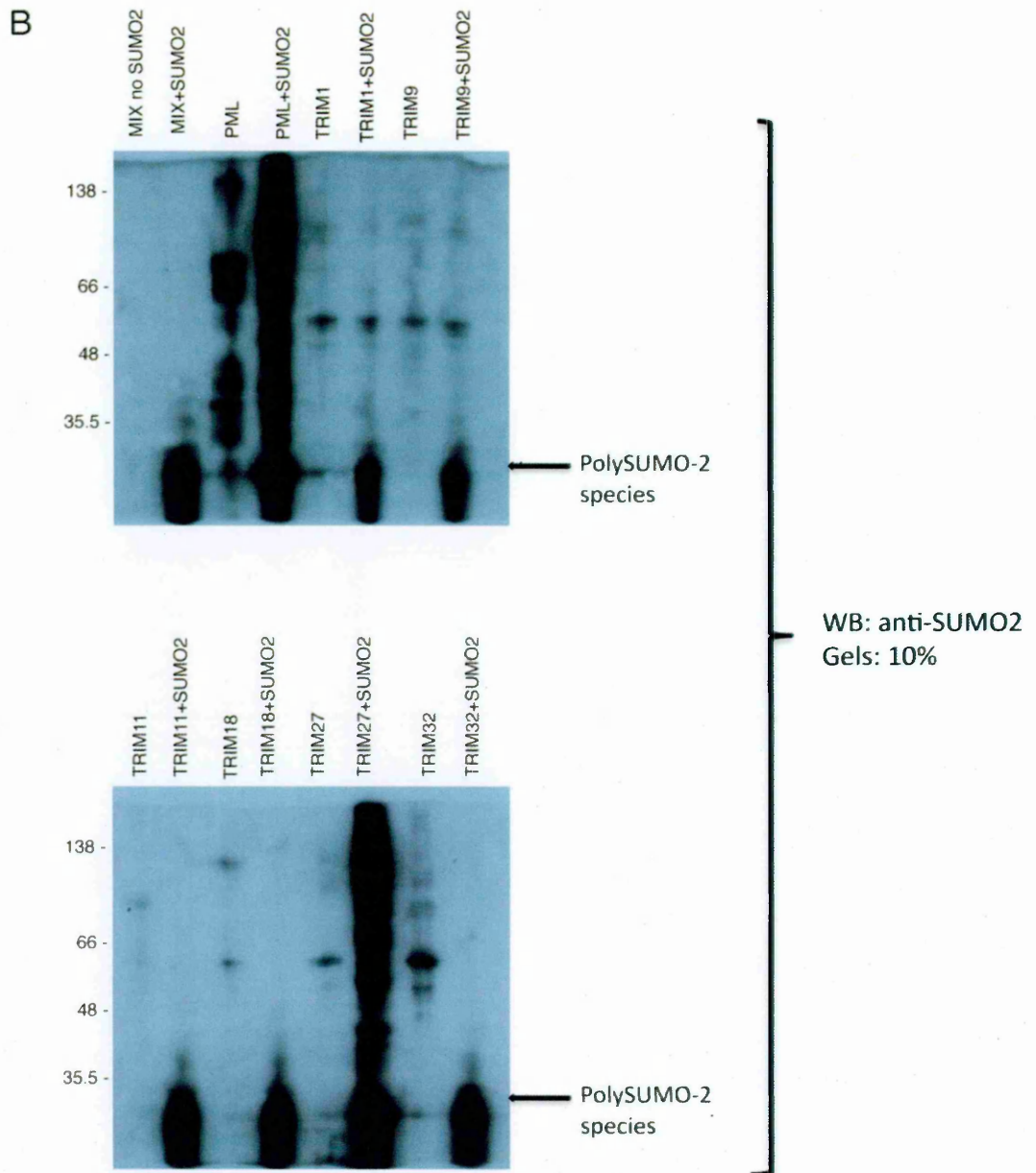
The above 6 MBP-TRIM proteins were incubated in the reaction mixture as indicated above. The immunoblot analysis of the reaction products using antibodies against SUMO1 revealed the presence of poly-SUMO1 species when the recombinant MBP-TRIM protein was added to the reaction mixture (**Figure 29a**). Interestingly, the immunoblot analysis of the reaction products against SUMO2 revealed the presence of the poly-SUMO2 species only when the recombinant MBP-TRIM27 was added to the reaction mixture (**Figure 29b**). In both cases GST-PML was used as experimental control.

Thus, taken together these preliminary data further demonstrate that TRIM proteins can participate in the SUMOylation pathway in a very specific manner acting either as substrate or as E3 SUMO ligase or both.

Figure 29



In vitro SUMOylation assays. A) MBP-TRIM proteins (TRIM1, TRIM9, TRIM11, TRIM18, TRIM27, TRIM32) were tested through *in vitro* SUMOylation experiments (SUMO1). As control, the assay was performed without SUMO1 peptide (lanes with the TRIM indicated). Immunoblot with anti-SUMO1 antibody to detect the poly-SUMOylated species is shown. GST-PML is used as experimental control. The experiment was repeated four times in the same order reported in the picture above that is representative of the results obtained.



B) MBP-TRIM proteins (TRIM1, TRIM9, TRIM11, TRIM18, TRIM27, TRIM32) were tested through *in vitro* SUMOylation experiments (SUMO2). As control, the assay was performed without SUMO2 peptide (lanes with the TRIM indicated). Immunoblot with anti-SUMO2 antibody to detect the poly-SUMOylated species is shown. GST-PML is used as experimental control. The experiment was repeated four times in the same order reported in the picture above that is representative of the results obtained.

CHAPTER 5: DISCUSSION

In my study I demonstrated that TRIM proteins are able to interact with Ubiquitin Conjugating Enzyme (UBE2) and are therefore likely to be implicated in the ubiquitylation process acting as a class of Ubiquitin Ligase or E3. I have also found that the majority of the TRIM proteins tested interact with UBE2 enzymes with a defined specificity that is maintained in their ability to act as E3 Ubiquitin ligases. Moreover, I have examined the involvement of TRIM proteins in the SUMO pathway, the best known among the Ubiquitin-like pathways (UBLs), and I have also demonstrated that TRIM proteins might partake in the process either as SUMO substrate or as E3 SUMO Ligases or both.

A direct interaction between UBE2 and E3 enzymes is required for the ubiquitin ligase reaction. However, the modest affinity and transient nature of E2/E3 complexes pose additional technical challenges for the identification of E2/TRIM pairs. Standard techniques based on affinity purification of bait protein, such as co-immunoprecipitation, rarely succeed due to the weak affinity of the complexes.

Co-immunoprecipitation is a very useful method to co-purify interacting protein partners. The success of this strategy depends on the availability of good-quality antibodies directed against the target protein. In addition, since this procedure involves a single purification step, this limits the use of this strategy for protein present in very low abundance. A strength of the co-immunoprecipitation strategy is, however, that proteins associated *in vivo* are copurified in a rapid and simple manner. Therefore, co-immunoprecipitation remains a rigorous method to validate the physiological significance of protein interaction. Consistent with the co-immunoprecipitation technique, pull-down assays should be used both to confirm protein-protein interactions predicted by other methods (i.e. co-immunoprecipitation, yeast two hybrid) and as initial screening assay for purifying unknown protein-protein

interactions. Pull-down assays are a form of affinity purification and are very similar to the co-immunoprecipitation except that a bait protein is used instead of an antibody. The most important limitation in the usage of the pull-down assays is the nature of the protein-protein interactions. Indeed, stable protein-protein interactions are **easy** to be isolated because the complex does not disassemble over time. Strong, stable protein complexes can be washed with high-ionic strength buffers to eliminate any false-positive results. If the protein-protein interaction is a transient one like E2/E3 pairs, the specific interaction is difficult to identify because the complex may dissociate during the assay and it could generate false negative interactions.

Recently, a new strategy for protein complex purification and for identification of interacting protein partners has been developed: the Tandem Affinity Purification (TAP). TAP involves the fusion of a protein tag to the target protein and its expression in host cell or organism. The purified material can then be fractionated on a gel, and co-purified proteins can be identified by mass spectrometry or Edman degradation. TAP strategy combines several advantages of the standard biochemical purification and the co-immunoprecipitation strategies like rapid, selective, and efficient recovery of the *in vivo*-associated target complex from the extract. Although the TAP method is broadly applicable, there are some important limitations. First, a functional TAP-tagged protein must be made. For some protein, tagging at both the amino and the carboxyl termini of the protein may affect its activity. Second, some proteins may contain an endogenous TEV protease cleavage that may interfere with protein purification. However, to better characterize the interactions of purified proteins, many techniques have been developed. Among these the label-free of protein-protein interaction at solid surface by Surface Plasma Resonance (SPR) is the most powerful. The most important advantage of this technique is the measurement in real-time of the kinetics of ligand-receptor interactions that facilitates SPR's application in label-free quantitative immune-assay techniques for proteins and small analytes, in

conformational studies of protein as well as in the real-time association-dissociation measurements of ligand-receptor interactions. However, SPR biosensors usually lack the sensitivity to detect the interaction of protein with small ligand and due to its inability to handle many samples simultaneously, they are unsuitable for use in high throughput applications.

Indeed, to screen a large number of protein-protein interactions, Yeast Two Hybrid and Protein Fragment complementation (PCA) are the elective methods. In particular, Y2H was inspired by the modular structure of transcription factors containing a DNA-binding domain (DBD) and a transcription activation domain (AD). Splitting the DBD and the AD inactivated the transcription factor, while its function could be restored fusing DBD and AD to two interacting proteins. The Y2H utilizes this effect to identify protein interactions by fusing a “bait” protein to a DBD and potential interaction partners (“prey” proteins) to the AD. Numerous variations of Y2H have been developed including systems with several reporter genes, one-hybrid and three-hybrid systems for identifying proteins interactions with DNA and RNA (Fashena, Serebriiski et al. 2000; Causier 2004). Even if the Y2H approach has many advantages like high specificity and quickness, it also has many disadvantages like: small overlap between Y2H experiments; the use of sequence chimeras can impose difficulties since fusion can change the structure of a target protein. In addition, protein folding and posttranslational modifications can differ between yeast and other organisms. The classical Y2H systems are limited to protein interactions in the nucleus; thus interactions involving proteins integrated into or anchored to the plasma membrane are barely accessible. This dilemma was resolved by extending the Y2H approach to the PCA assays that were implemented in the split ubiquitin technique. Other enzymes, such as adenylate cyclase or an anthranilate isomerase have been used for split-protein-based screening of protein interactions in different organisms. A common feature of these approaches is the indirect and time-shifted response, as they rely on the transcription of the

reporter gene. Another general approach related to PCA is based on protein splicing and has been applied to several reporter proteins, such as GFP and firefly luciferase. Although Y2H and PCA are potent techniques for identifying interactions, the real-time monitoring and localization of protein interactions in living-cells requires a direct spectroscopic investigation. To date, the most powerful technique for the direct spectroscopic detection and monitoring of protein interactions in living cells is fluorescence resonance energy transfer (FRET). The detection of protein interactions in living cells by FRET can be carried out conveniently and with high throughput by flow cytometry. However, FRET technique is limited by the relatively high background of cellular autofluorescence as well as by the direct excitation of the fluorescence acceptor, which frequently biases the interaction experiments. Since the high number of false positive generated by Y2H, PCA or FRET techniques, all the protein-protein interactions detected need to be confirmed through other techniques like co-immunoprecipitation, pull-down or TAP.

Thus, taken together all the information about the techniques generally used to detect protein-protein interactions, I decided to screen all the putative interactions between TRIM proteins and UBE2 enzymes through the Yeast Interaction Mating technique since it has been somewhat more successful in identifying E2/E3 pairs, presumably because a positive read-out can be obtained even for transient interactions. Indeed, two large-scale Y2H screens for E2 partners have been reported. A screen that utilized full-length E2s as bait against circa 150 RING-protein preys yielded putative partners for all but two of 39 E2s (Cdc34 and Birc6) and for approximately 90 of the E3 preys (Markson, Kiel et al. 2009). Instead, a screen of over 250 RING domain preys with 36 E2 Ubc domain baits failed to identify a binding partner for ten E2s that are known to conjugate Ub and fewer than half the RING domains returned a positive E2 interaction (van Wijk, de Vries et al. 2009). The different outcomes in the screens may be due in part to the use of full-length

versus specific E2 and E3 domains, consistent with emerging evidence for non-canonical E2/E3 interactions. However, the requirement of some RING E3s to exist as heterodimeric or multi-component complexes may further affect the attainable yield in a yeast Interaction Mating screen. For example, in a targeted Y2H screen aimed at identifying the human E2s that interact with the heterodimeric RING E3, BRCA1/BARD1, a bait construct in which the RING domains of BRCA1 and BARD1 were fused into a single polypeptide that folds correctly into the E3-active structure identified ten E2s that interact with BRCA1/BARD1 (Christensen, Brzovic et al. 2007). Screens using baits comprised solely of the BRCA1 RING failed to identify any E2s that have been shown biochemically to transfer Ub (Markson, Kiel et al. 2009; van Wijk, de Vries et al. 2009). Within a Y2H screen, the E2 may or may not be conjugated to Ub, depending on whether the endogenous yeast E1 is able to charge the E2 of interest among other factors. Therefore, an issue that may contribute to a failure to identify E2s for an E3 in a Y2H experiment is that Y2H studies may only screen for interactions between an E3 and a free E2, although the functionally relevant interaction involves the E2~Ub conjugate. There are examples of E3s that show detectable binding only to an E2~Ub. For example, SspH2 (a bacterial protein with E3 ligase activity) binds only to an activated E2~Ub conjugate and not to the individual components (Levin, Eakin et al. 2010). It remains to be seen if this feature will be unique to bacterial E3s that have evolved *via* convergent evolution to work with host E2 enzymes or whether there are eukaryotic E3s that only bind to E2~Ub species. In any case, these examples demonstrate the complicated and context-dependent nature of E2/E3 interactions that confound the ability to identify them.

In all the seven Interaction Mating screens performed, I could observe that the majority of the TRIM proteins fulfil this rule and I detected more than 100 interactions between UBE2 and TRIM proteins. As already observed with other ligases, in most cases TRIM proteins interact with more than one UBE2

enzyme and *vice versa* (van Wijk, de Vries et al. 2009). I believe that the TRIM proteins that cannot interact with any of the UBE2 proteins tested, might not be expressed in the yeast or may assume an improper conformation that may hide the DNA Binding or B42 domains. Consistently, the majority of the TRIM proteins that present no interactions with the UBE2 enzymes cannot homo-dimerize (Reymond, Meroni et al. 2001). It is also possible that in some cases TRIM proteins might not be able to interact with UBE2 at all or that they could interact with UBE2 enzymes not used in my experiments. Moreover, since for some TRIM proteins have been reported their ability to form hetero-complexes, it might be that hetero-interactions is an important requirement to act as E3 Ubiquitin Ligase.

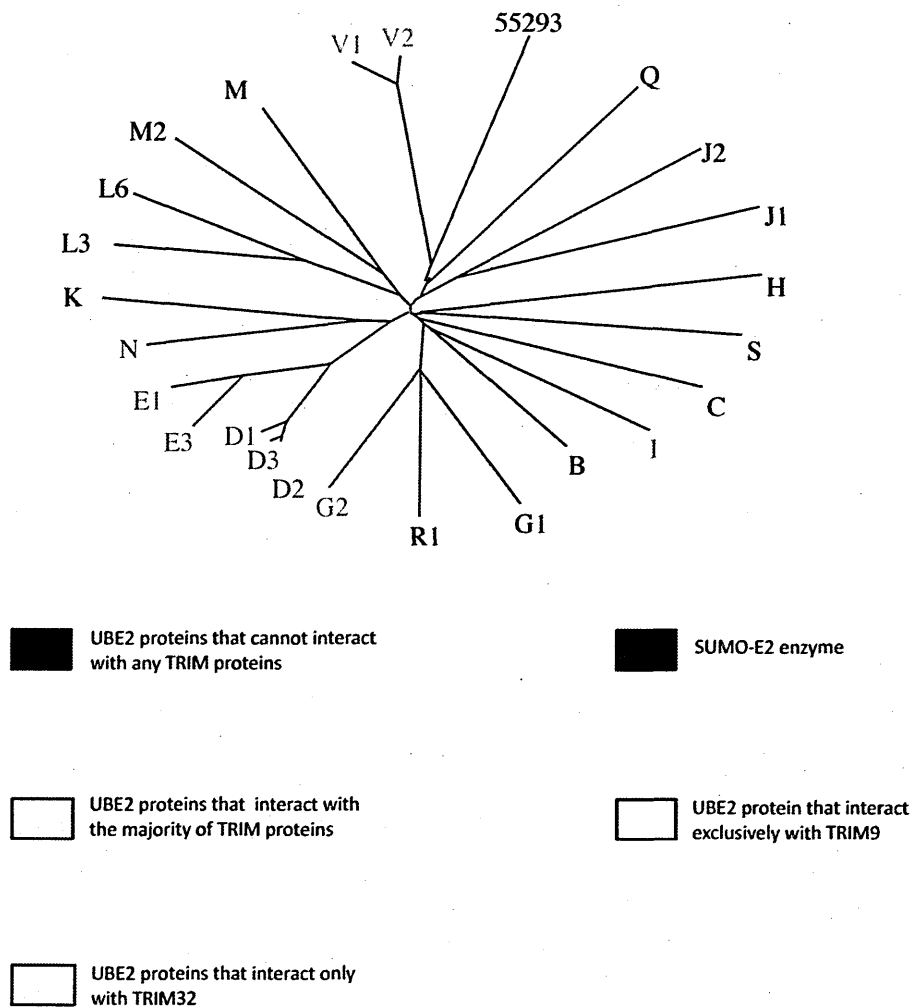
Moreover, I have demonstrated that the RING domain mediates the interaction between the TRIM proteins and the UBE2 enzymes. I tested TRIM18 and TRIM32 mutants carrying single domain deletions (RING, B-box, Coiled-coil, and RFP-like) and I demonstrated that deletion of the RING finger results in the loss of interaction with UBE2 proteins. Furthermore, an isolated RING finger domain, but not RFP or BB-CC domains, could interact with the UBE2 enzymes, confirming that the RING motif is necessary and sufficient for the interaction with the UBE2 proteins. The concept that this is the domain offering the surface for direct E2/E3 interaction is reinforced by the lack of UBE2 binding observed with the naturally occurring RING-less TRIM proteins (Sardiello, Cairo et al. 2008). My data also suggest that the B-boxes are not directly involved in this interaction. B-box1 and B-box2 are zinc-binding domains found within the tripartite module (Sardiello, Cairo et al. 2008) that assemble to form RING-like structure. Given this resemblance, it has often been speculated that the B-boxes might also interact with E2 enzymes. Recently, Han et al. (2011) have demonstrated that MID1/TRIM18 B-boxes support RING role in the ubiquitylation process (Han, Du et al. 2011). In particular, they observed that MID1/TRIM18 B-box1 have a key role because it appears to amplify the E3 activity of the RING domain

presenting additional lysine residues to be mono-ubiquitylated (Han, Du et al. 2011). It is tempting to speculate that B-boxes have originally evolved to function as E3 ligases but now they may support RING domain.

As a general rule, the majority of the TRIM proteins tested preferentially interact with a subset of the 26 UBE2 enzymes, which is constituted by: UBE2D1, UBE2D2, UBE2D3, UBE2D4, UBE2N, UBE2E1, UBE2E2, and UBE2E3. On the other hand, they do not interact with UBE2L3/UBE2L6 enzymes indicating that the interaction between the TRIM proteins and the UBE2 enzymes is specific. Indeed, figure 5A shows a phylogenetic tree of 20 of the UBE2 proteins utilized in my experiments, which indicates that the subset of the UBE2 proteins that interact with the majority of the TRIM proteins is constituted by UBE2 enzymes that are evolutionary close to each other. The only exception is the UBE2I that is not closely related to the subset of the UBE2 enzymes indicated above. This UBE2 interacts with almost all TRIM and, since the UBE2I is the specific UBE2 involved in the SUMOylation process, this interaction may indicate that many TRIM proteins could be involved in this Ubiquitin-like (UBLs) process or it could be less specific because SUMO modified UBE2I, in yeast, may bind to SUMO Interacting Motif in the TRIMs. Instead, the UBE2L3/UBE2L6 enzymes, which do not interact with TRIM proteins, are distant in the phylogenetic tree from the subset indicated above (**Figure 30**). My data support a model in which the RING TRIM proteins that interact with the UBE2L3/UBE2L6 enzyme cannot interact with the UBE2D and E family and *vice versa* (Martinez-Noel, Muller et al. 2001).

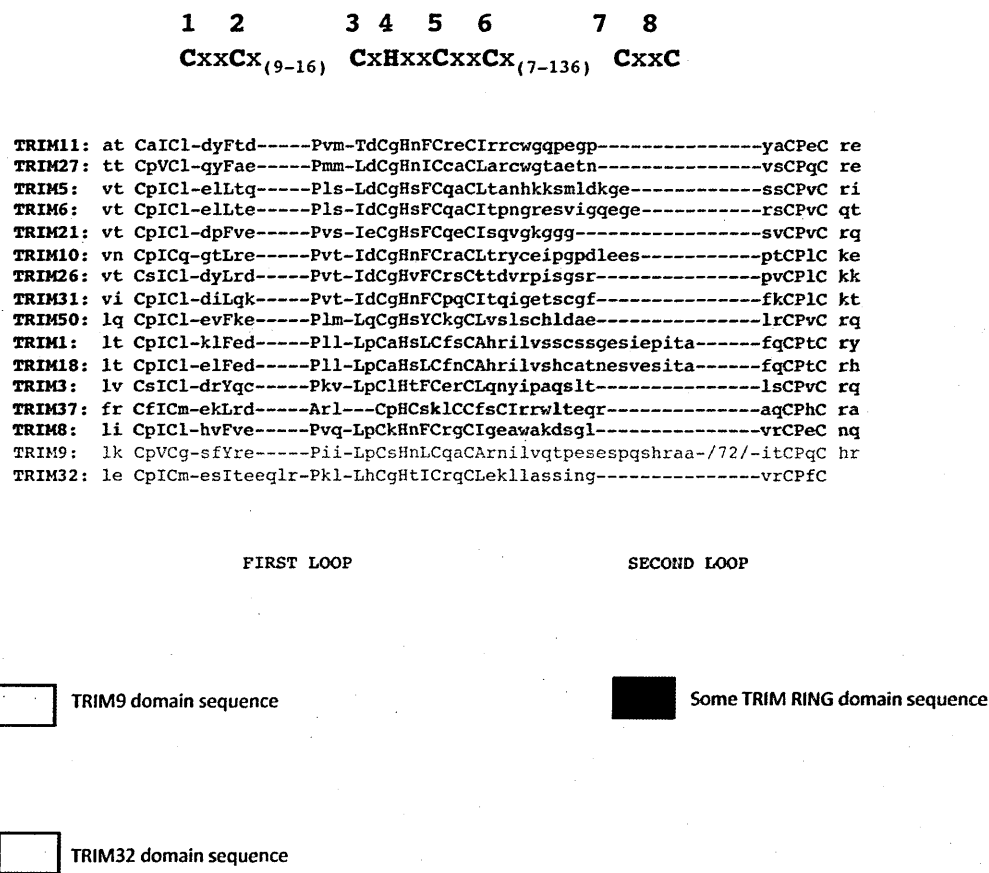
In my experiments I also observed two important exceptions: TRIM9 and TRIM32 that are the only ones that can interact with UBE2G2 and UBE2V1/UBE2V2, respectively. Given that the RING domain is involved in the interaction between these TRIM ones and the UBE2 proteins indicated above, I aligned the RING domain primary sequences of some TRIM proteins (**Figure 31**).

Figure 30



A phylogenetic tree produced upon the alignment of the 20 UBE2 proteins tested. The majority of the TRIM proteins interact with a subset of UBE2 enzymes, i.e. UBE2D1, UBE2D2, UBE2D3, UBE2N, UBE2E1, UBE2E3, and UBE2I. These UBE2 proteins are very close within the tree and therefore are more similar to each other. None of the TRIM proteins that interact with the subset indicated above could interact with UBE2L3/UBE2L6 that seem to diverge from the subset of UBE2 indicated above.

Figure 31



I could notice that TRIM9 and TRIM32 RING domains present peculiar sequence patterns. TRIM9 RING domain presents a second loop which is longer than the second loop of the other TRIM proteins; on the other hand both the first and the second loop of TRIM32 are shorter than the other ones. This observation supports the specificity of TRIM-UBE2 interactions determined by the RING sequence that probably translates into a slightly different structure.

I confirmed that the selected TRIM proteins behave *in vivo* as RING-dependent ubiquitin ligases with a major involvement in proteasome-mediated degradation. I also assessed the *in vitro* ubiquitin E3 activity of TRIM1, 9, 11, 18, 27, and 32 in the presence of the specific UBE2 enzyme(s) they interact with. The binding specificity between TRIM proteins and UBE2 enzymes is perfectly translated in the ability to function as E3 ligases. I now provide a direct proof of TRIM ubiquitin ligase activity *in vitro* and of the identity of the UBE2 enzymes they use for this function: D1-3, E1, N and, in the case of TRIM11 also J2, but not L3 and L6. Moreover, I assessed for the first time *in vivo* and *in vitro* E3 activity for TRIM1 and its attitude to use D1-3, E1 and N classes of UBE2 to exert this function. In the case of TRIM27, despite many biochemical findings as transcriptional and signalling regulator (Shimono, Murakami et al. 2000) few data on the E3 activity are available. Interestingly, differently from a recent report, I could observe the inability to interact and function with D2 (Gillot, Matthews et al. 2009).

One of the most specific functional interactions I assessed is between TRIM9 and UBE2G2. Interestingly, these two genes that are specifically expressed in the embryonic and adult nervous system further supporting their specific interaction (Berti, Messali et al. 2002) (www.genepaint.org). In the case of UBE2G2, structural and biochemical studies demonstrated that for its specific interaction with the gp78 E3 ligase an additional domain is required (Chen, Mariano et al. 2006) I still do not know if other regions of the TRIM9

protein are implicated in these specific interactions. However, its RING sequence is peculiar as it presents an extended second loop if compared to the class D and E interacting TRIM proteins (**Figure 31**) (Sardiello, Cairo et al. 2008). I could observe that overexpression of TRIM9 within the cells induces a relocalisation of UBE2G2 that is recruited in a specific manner in the TRIM9 cytoplasmic bodies. TRIM9 is a neuron specific component of a SNARE complex associated with synaptic vesicle release control (Li, Chin et al. 2001). UBE2G2 is one of the two E2 enzymes involved in endoplasmic reticulum-associated degradation (ERAD) (Chen, Mariano et al. 2006). It is tempting to speculate that TRIM9 might cooperate with UBE2G2 in the ERAD control of membrane-associated synaptic SNARE proteins destined to the secretory pathway.

TRIM32 ubiquitin E3 activity has been already reported on several physiological substrates (Albor, El-Hizawi et al. 2006). Consistent with my results, the above activity requires the presence of D and E classes of UBE2 enzymes (Albor, El-Hizawi et al. 2006). Moreover, I added information on the activity with UBE2N to these data. Besides binding with the D, E and N types, I found that TRIM32 interacts in a very specific manner with UBE2V1 and V2, which are catalytically dependent on the UBE2N for their function. Indeed, I found that TRIM32 is a more efficient E3 ligase in the presence of UBE2N/V1 than with UBE2N alone. Interestingly, so far no direct interaction has been reported between an E3 ligase and either UBE2V1 or V2. My data clearly show that not all the TRIM proteins observed to bind UBE2N also interact with UBE2V1 and V2 stressing the peculiar ability of TRIM32 in these interactions. The immunofluorescence experiments show that TRIM32 and the aforementioned UBE2 enzymes change their reciprocal localisation within HeLa cells further suggesting that also *in vivo* TRIM32 may take advantage of the use of UBE2V1 and V2 for its activity. Noteworthy, the hetero-dimer UBE2N/V1 is specifically involved in NFkB pathway, the same

in which TRIM32 also participates through its ability to control Piasy degradation (Albor, El-Hizawi et al. 2006-).

TRIM proteins' involvement in different cellular process such as regulation of cell cycle and division, transcriptional regulation, differentiation and development, apoptosis, DNA repair, regulation of the immune and inflammatory responses have been reported (Meroni and Diez-Roux 2005). Since many of these cellular processes have as critical regulators the Ubiquitin-like proteins (SUMO, ISG15, Nedd8 and Atg8), my study also provided preliminary data about TRIM proteins' involvement in SUMOylation pathway. I tested interaction between TRIM proteins and UBE2I, which is the exclusive SUMO-Ubiquitin Conjugating Enzyme, again by the Interaction mating technique. I found that the majority of the TRIM proteins tested interact with UBE2I/Ubc9 and that RING domain is not closely involved in TRIM/UBE2I interaction. Recently Y Chu and X Yang (Chu and Yang 2011) have indicated TRIM proteins as a new class of SUMO E3 Ligase. In their study they also demonstrated that TRIM SUMO E3 activity relies on both the RING domain and intact B1- and B2-boxes. Although speculative, it is possible that TRIM protein may also attach ubiquitin and SUMO on the same protein target simultaneously or sequentially by using different protein domain.

SUMOylation differ from both Ubiquitylation and other Ubiquitin-like pathways because the UBE2I can attach polySUMO1/2/3 chains to the substrate without E3 enzymes, albeit with lower efficiency. I further performed *in vitro* SUMOylation assay on a selection of TRIM proteins whose interaction with UBE2I was previously confirmed by the MBP-pull down assay. I found that all TRIM proteins tested (TRIM1, TRIM9, TRIM11, TRIM18, TRIM27, TRIM32) act as both/either SUMO ligase and/or substrate by adding SUMO1 to the reaction mixture; on the contrary only TRIM27 act as either SUMO ligase or substrate or both with SUMO2. These data are supported by TRIM27 specific binding to PML/TRIM19, that has been

proposed to have an E3 SUMO1/2 ligase activity located to its RING domain. Moreover, between the subgroup of TRIM proteins selected, TRIM27 is the only one that localizes in the nucleus and, in particular, in the Nuclear Bodies (NBs) where SUMO2 is abundant. It is tempting to speculate either that TRIM proteins may act as E3 Ubiquitin Ligase whose activity is regulated by SUMOylation or that TRIM proteins may act as a novel class of E3 Ubiquitin and SUMO Ligase involved in the regulation of many cellular processes.

Conclusively assessing the *in vivo* role of the TRIM proteins, however, will require further efforts. Interaction with more than one UBE2 enzymes may underline their consecutive usage, especially when D and E classes are concerned, and the formation of different chains of specific linkage. Thus, a single TRIM may have dual or more E3 activity with a consequent involvement in different cellular process. As example, my screens underline the ability of TRIM32 to bind both UBE2D enzymes that are involved in K48 poly-ubiquitin chain formation, and UBE2N/V1-UBE2N/V2 complexes that are both involved in K63 poly-ubiquitin chain formation. It is presumable a dual role for TRIM32: on a hand regulation of some cellular processes through protein specific degradation, on the other hand activation of specific intracellular pathways (i.e. DNA repair and activation of NF- κ B). Candidate E2 enzymes identified as interacting with TRIM32 must be followed up *in vitro* ubiquitylation study using ubiquitin mutants (i.e. K48 and K63 ubiquitin chain mutants) to better investigate TRIM32 biological role and to confirm a different effect on its E3 function. Of course, it could be useful repeating the same analysis for all the TRIM proteins selected for my PhD work.

It is well assessed that TRIM E3 ligases function in diverse processes in the cell. The biological role of many TRIM proteins, however, is still obscure and an important aspect in understanding E3 ligase function is to identify the biological context in which they function and their physiological substrate(s). Even if my work gives an important indication for putative TRIM biological role thanks to their selective usage of specific UBE2 enzymes, it will be

necessary to identify putative substrate(s) through Yeast Two hybrid or RNAi screen. Consequently, it must verify if TRIM proteins preferentially use the UBE2 identified in my screening for either auto-ubiquitylation or substrate ubiquitylation or both to better understand mechanism inside TRIM/E2 interactions.

Detailed structures have been solved for E2/E3 complex and amino acid residues responsible for these interactions have been identified. Moreover, more detailed information are available regarding UBE2 amino acid residues than E3's ones. It might be interesting to assess putative TRIM/UBE2 complex structure through Homology Modeling procedure comparing TRIM/UBE2 experimental three-dimensional structure with E3/E2 complexes already solved. Taken together all the data presumable collected may give important indication for the design of drugs that might block the ubiquitylation pathway in a selective way. Since the involvement of TRIM proteins in many pathological conditions from cancer to immunological disease, the modeling of drugs that are able to block in a specific way the pathway in which TRIM proteins are involved could give a good effort in the treatment of the specific diseases.

Finally, the screening of TRIM proteins for their cadre of interacting E2s will lead to further insight into the mechanisms of protein ubiquitylation. Some foreseeable areas that will benefit include: identification of more E3s that function with the ubiquitin-like proteins, development of predictive models guiding E2 selection by an E3, and ideally, the ability to thoroughly investigate E3-dependent substrate ubiquitylation.

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